FINAL REPORT

Study Title

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH AEROSOL AND LIQUID DOSING, TWO TIME EXPOSURES AND HISTOLOGY
Using CG and BJ

Authors

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Study Completion Date

October 4, 2005

Performing Laboratory

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Study Number

05AD40, AD42.350066

Laboratory Project Number

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10 (d) (1) (A), (B), or (C).

Company:

Company Agent: ______________________________ Date: ________________________

______________________________ Signature:_____________________
Title

STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity and Permeability Assay with Aerosol and Liquid Dosing, Two Time Exposures and Histology of the test substance, CG and the reference substance, BJ, was conducted in compliance with U.S. EPA GLP Standards 40 CFR 160 with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test substance have not been determined by the testing facility.
>
> The processing of the corneas and preparation of the slides by Pathology Associates, Inc. was not performed under the GLP guidelines.

Study Director ______________________________ Date __________________
John W. Harbell, Ph.D.

Submitter: ______________________________ Date __________________

Sponsor's
Representative: ______________________________ Date __________________

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Aerosol and Liquid Dosing, Two Time Exposures and Histology

Study Number: 05AD40, AD42.350066

Study Director: John Harbell, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	05-Jul-05	05-Jul-05	05-Jul-05
Final Opacity Measurement	05-Jul-05	06-Jul-05	06-Jul-05
Histology – Negative Control and 05AD40-I 10 minute exposure	08-Sep-05	08-Sep-05	19-Sep-05
Draft Report and Data	19-Sep-05	19-Sep-05	19-Sep-05
Final Report	04-Oct-05	04-Oct-05	04-Oct-05

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

Date

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SIGNATURE PAGE

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH AEROSOL AND LIQUID DOSING, TWO TIME EXPOSURES AND HISTOLOGY

Initiation Date:	June 30, 2005
Completion Date:	October 4, 2005
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Sponsor's Representative:	
Testing Facility:	Institute for In Vitro Sciences, Inc. 21 Firstfield Road, Suite 220 Gaithersburg, MD 20878
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Histology Evaluation Performed by:	John W. Harbell, Ph.D.

TEST/REFERENCE SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions *
05AD40	CG	clear colorless non-viscous liquid	6/2/05	room temperature
05AD42	BJ	clear colorless non-viscous liquid	6/2/05	room temperature

* - Protected from exposure to light

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH AERSOL AND LIQUID DOSING, TWO TIME EXPOSURES AND HISTOLOGY

SUMMARY

The ocular irritancy of CG were evaluated in the Bovine Corneal Opacity and Permeability (BCOP) Assay. BJ served as a reference substance. Positive and negative control materials were tested concurrently in the study. The test article, CG, was exposed to the corneas using the aerosol delivery procedure and the direct instillation procedure using 750µL per cornea (normal BCOP exposure method). The reference substance, BJ, and the positive and negative control materials were exposed to the corneas using the direct instillation procedure using 750µL per cornea. Each treatment condition was applied to three corneas. The corneas were exposed to the test and reference substances for periods of 3 and 10 minutes followed by a post-exposure incubation of 2 hours. After determination of the final opacity measurements and assessment of the penetration of fluorescein, each cornea was fixed for histological evaluation.

Summary Table
BCOP Results of the Test and Reference Substances

Assay Date	IIVS Test/Reference Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score	pH
7/5/05	05AD40-A	CG	Neat	3 minutes	3.3	0.008	3.5	12.0
				10 minutes	3.7	0.014	3.9	
	05AD40-I	CG	Neat	3 minutes	23.0	0.148	25.2	
				10 minutes	29.0	1.274	48.1	
	05AD42-I	BJ	Neat	3 minutes	4.3	0.258	8.2	12.5
				10 minutes	29.0	1.704	54.6	

A- Aerosol method of exposure.
I- Instillation method of exposure.

The response of the positive control, neat ethanol, was within the normal range and therefore the assay was considered valid according the acceptance criteria of the protocol.

Histological evaluation summary: Corneas exposed to CG by the aerosol route for three minutes showed only minimal changes and these changes were confined to the surface epithelium. Similar exposure for 10 minutes produced somewhat deeper damage into the upper epithelium. There was also some slight stromal swelling directly under the epithelium. Keratocyte changes were minimal. Corneas exposed to CG by the instillation route for three minutes again showed only changes confined to the epithelium. The increased opacity score was the result of the surface coagulation. The stroma was undamaged. Exposure for 10 minutes produced deeper epithelial damage but only minimal changes in the upper stroma. The deeper stroma was normal. These data suggest that test article CG would produce minimal damage by

the aerosol route and only slight damage by the instillation route. The reference substance BJ was exposed to the corneas by the installation route. The corneas treated for 3 minutes showed some coagulation in the surface epithelium and some increased cytoplasmic vacuolization in the deeper epithelium. The upper stroma showed moderate vacuolization and a moderate increase in keratocytes with enlarged nuclei and cytoplasmic eosinophilia. These stromal changes may reflect the reduction of epithelial barrier integrity that allowed water to enter the stroma rather than direct action of the reference substance on the stroma. The 10-minute exposure produced appreciable epithelial damage and resulting loss of epithelial barrier integrity. Stromal changes included marked collagen matrix vacuolization and a marked increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia. Again, these keratocyte changes may reflect the impact of stromal swelling rather than direct action of the reference substance on the cells themselves[1]. These corneas also showed some loss of endothelial cells. These data suggest that the reference substance has more eye irritation potential than does the test substance when compared using the instillation route of exposure.

[1] Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. ALTEX 42(Special Issue):236.

INTRODUCTION

The purpose of this study was to evaluate the potential ocular irritancy of the test substance, CG, in the Bovine Corneal Opacity and Permeability (BCOP) Assay. The assay procedures generally followed those described by Sina *et al.* (1995)[2]. The test substance, CG, was exposed to the corneas using the aerosol delivery procedure and the direct instillation procedure using 750µL per cornea (normal BCOP exposure method). The reference substance, BJ, and the positive and negative control materials were exposed to the corneas using the direct instillation procedure using 750µL per cornea. This study was performed at the Institute for In Vitro Sciences, Inc. (IIVS), 21 Firstfield Road, Suite 220, Gaithersburg, MD, 20878. The protocol was signed by the Study Director on June 30, 2005, and the assay was begun on July 5, 2005. At the conclusion of the opacity and permeability measurements, the corneas were fixed and sent out for histological preparation. The slides of the test substance and control treated corneas were evaluated at IIVS. The in-life phase of the study concluded, following examination of histological slides, on September 9, 2005.

[2] Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

MATERIALS AND METHODS

Test and Reference Substance Preparation

As instructed by the sponsor, the test and reference substances were administered to the test system without dilution.

Test and Reference Substance pH Determination

The pH values of the test and reference substances were determined using pH paper (EMD Chemicals Inc./ EM Science). Initially, the test and reference substances were added to 0-14 pH paper with 1.0 pH unit increment to approximate a narrow pH range. Next, the test and reference substances were added to 7.5-14 pH paper with 0.5 pH unit increments, to obtain a more precise pH value. The pH values obtained from the narrower range pH paper are recorded in Table 1.

Assay Controls

The positive control used in this study was neat ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TRUETH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at 32 ± 1ºC for a minimum of 1 hour.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer.

Three corneas, whose initial opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test substance number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

<u>Method for Testing Liquid or Surfactant Materials</u>

The liquid test substance, CG, and the reference substance, BJ, were tested neat. The test substance, CG, was tested using both the aerosol and instillation exposure methods. The reference substance, BJ, and the assay controls were tested using only the instillation method of exposure. For aerosol exposure, a dose assist device was prepared from a 50-mL conical tube (please see protocol section 7.6.1. for specific details [page 47 of this report]). With the chamber held in a vertical position, an approximately one second burst of aerosol spray was directed straight at the center of the exposed cornea (anterior chamber window removed) down the conical tube. The chamber was then rotated back to a horizontal position and the window was replaced insuring adequate coverage of the cornea with the test substance. For instillation exposure, an aliquot of 750 µL of the test substance, reference substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. Two sets of three corneas, one set per exposure method, were incubated in the presence of the test substance at either room temperature or 32 ± 1ºC for 3 minutes. Two additional sets of three corneas, one set per exposure method, were incubated in the presence of the test substance at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the reference substance at 32 ± 1ºC for 3 minutes. A second set of three corneas was incubated in the presence of the reference substance at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the negative control at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the positive control at 32 ± 1ºC for 10 minutes. After the 3 and 10-minute exposure times, the assay control, test substance, or reference substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control material, or test or reference substances. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1ºC. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 µL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices *V*max kinetic microplate reader. If the OD_{490} value of a control material or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 µL sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

The fixed corneas were transferred to Pathology Associates, A Charles River Company (Frederick, MD) for embedding, sectioning, and staining. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin block so that a section of each half could be cut and placed on a single slide. Each slide was then stained with hematoxylin and eosin. Slides were returned to IIVS for evaluation.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD_{490} for the blank wells was calculated. The mean blank OD_{490} was then subtracted from the raw OD_{490} of each well (corrected OD_{490}). Any dilutions that were made to bring the OD_{490} readings into the linear range of the platereader (OD_{490} should be less than 1.500), had each diluted OD_{490} reading multiplied by the dilution factor. The final corrected OD_{490} of the test and reference substances and the positive control was then calculated by subtracting the average corrected OD_{490} of the negative control corneas from the corrected OD_{490} value of each treated cornea:

Final Corrected OD_{490} = (raw OD_{490} – mean blank OD_{490}) – average corrected negative control OD_{490}

The mean OD_{490} value of each treatment group was calculated by averaging the final corrected OD_{490} values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for the test and reference substances. Table 2 summarizes the opacity, permeability and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 40.1 to 65.1), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

Table 1
BCOP Results of the Test and Reference Substances

Assay Date	IIVS Test/Reference Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score	pH
7/5/05	05AD40-A	CG	Neat	3 minutes	3.3	0.008	3.5	12.0
7/5/05	05AD40-A	CG	Neat	10 minutes	3.7	0.014	3.9	12.0
7/5/05	05AD40-I	CG	Neat	3 minutes	23.0	0.148	25.2	12.0
7/5/05	05AD40-I	CG	Neat	10 minutes	29.0	1.274	48.1	12.0
7/5/05	05AD42-I	BJ	Neat	3 minutes	4.3	0.258	8.2	12.5
7/5/05	05AD42-I	BJ	Neat	10 minutes	29.0	1.704	54.6	12.5

A- Aerosol method of exposure.
I- Instillation method of exposure.

Table 2
BCOP Results for the Positive Control

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score
7/5/05	Ethanol	10 minutes	32.0	1.285	51.3

Histological Evaluation

Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen in the cornea. This is not surprising as the test materials are applied topically to this “unprotected” epithelium. Each “layer” of the epithelium is scored for cell loss or damage. Figure 1 shows the structure of the epithelium from a control cornea (not from this study). Changes to the surface epithelium (squamous and upper wing cell epithelium) in the BCOP assay are usually not predictive of lasting corneal changes *in vivo.* The loss of the squamous and upper wing cell layers in the excised bovine cornea by surfactant-based test articles appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo.* This type of damage is typically reversible in the rabbit. Many types of surfactants (e.g., sodium lauryl sulfate) are expected to lyse these cells so that they are progressively lost from the epithelial surface. The positive control, ethanol, provides an example of a different type of damage. In this case, the surface squamous epithelial cells are coagulated and fixed in place. The coagulated tissue stains heavily with eosin (see Figure 6). In addition, the wing and basal cells are vacuolated and the adhesion between the basal cells and the basal lamina is disrupted. Such changes can lead to sloughing of the epithelium. Lesions in the deep wing cell and basal cell layers, either by cell lysis or coagulation, are associated with more damage *in vivo.* Much of the available data come from studies on surfactants and show that loss of the bovine corneal epithelium is predictive of some corneal opacity in the rabbit[3].

Special effort has been made to detect changes in the stromal elements of the corneas. Jester[4], Maurer[5,6] and others have shown for a range of chemical classes that depth of injury in the early hours after exposure can be predictive of the eventual degree and duration of the ocular lesions. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deep injury to the stroma has more serious consequences. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling within the stromal collagen matrix and loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected by the presence of vacuole-like "holes" in the organized collagen matrix. Their appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of these vacuoles may be seen in Figure 9 where the positive control exposure has induced some stromal swelling. The depth and degree of vacuolization can be indicative of the degree of injury to the cornea and/or penetration of the test article into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix vacuolization (swelling). Viable epithelium or endothelium will not only retard entry of the water but actively transport it out of the stroma. As the viability of epithelium declines, both the barrier and transport capacities are reduced. Thus, the amount of water accumulating in the upper stroma reflects the damage to the epithelium. When the full epithelial layer is lost over a section of stroma, stromal swelling can increase the stromal thickness by 50% or more over the control corneal stroma. Such changes are often observed when corneas treated with severe irritants are incubated for extended periods after exposure. Loss of the endothelium can also lead to

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[3] Gettings, SD, Lordo, RA, Hintze, KL, Bagley, DM, Casterton, PL, Chudkowski, M, Curren, RD, Demetrulias, JL, DiPasquale, LC, Earl, LK, Feder, PI, Galli, CL, Glaza, SM, Gordon, VC, Janus, J, Kuntz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food Chemical Toxicology** 34:79-117.

[4] Jester, J.V., Li, H.F., Petroll, W.M., Parker, R.D., Cavanaugh, H.D., Carr, G.J., Smith, B., and Maurer, J.K. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Invest Ophthalmol Vis Sci** 39:922-936.

[5] Maurer, J.K. and Parker, R.D. (1996) Light microscopic comparison of surfactant-induced eye irritation in rabbits and rats at three hours and recovery/day 35. **Toxicologic Pathology** 24:403-411.

[6] Maurer, J.K., Parker, R.D., and Jester, J.V. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

appreciable deep stromal swelling. The loss may result from test article penetration or mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test article exposure tends to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix vacuolization will be located in the deep stroma (just above Descemet's Membrane). In contrast, test article-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix vacuolization will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

In vivo, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through "scar" collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test article-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the beginning of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic eosinophilia. Harbell and Curren[7] have reported that mechanical removal of the corneal epithelium leads to marked stromal swelling (marked collagen matrix vacuolization). The keratocytes in the zone of marked swelling undergo changes as well. The nuclei become enlarged and the cytoplasmic staining is very eosinophilic. Thus, when test article-treated corneas show only this type of keratocyte change (with stromal swelling), the change may be the result of the stromal swelling rather than direct action of the test article on the cells.

Since depth of injury (both extracellular and cellular) to the stroma is important in the analysis, a means to describe the depth of injury is necessary as part of this analysis. Determining this depth is not always a straightforward process. Because of the topical application of the test article to the epithelium, one would expect that exposure to the stroma would progress from the area just under Bowman's Layer down through the stroma to Descemet's Membrane. There is no external inflammatory process *in vitro*, so one might also expect the progression of damage to follow the progression of exposure. That means that damage to the stroma should first appear close to Bowman's Layer. As the damage to the stroma increases, deeper layers might be involved. This implies that one would want to express damage as progressing from the anterior (Bowman's Layer) to the posterior (Descemet's Membrane) and express it as a fraction of the total stromal depth involved. However, collagen matrix vacuolization can (and often does) increase total stromal thickness. Thus, measurements of the depth of a stromal lesion can be complicated by the change in overall stromal thickness. To account for stromal swelling, this depth is actually estimated from the percentage of the stromal cross section that remained

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[7] Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. ALTEX 42(Special Issue):236.

undamaged (starting at the posterior border). For example, a cornea reported to show collagen matrix vacuolization to 30% depth would mean that 70% of the cross section of that cornea (starting at Descemet's Membrane) did not show vacuolization. For this report, depth of stromal damage is reported as the percentage of the normal corneal depth (cross-section) involved, starting from the anterior border (Bowman's Layer). It should be clearly understood that the percentage of the stromal depth is only an estimate developed by evaluating several fields in each cornea (where possible). The values are, by necessity, approximations of an average depth to which the lesion extended (e.g., collagen matrix vacuolization). It would be unwise to try to compare small differences in the reported depth. Rather, one should focus on broader bands of depth (e.g., upper, middle and deep stroma).

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Where little or no damage to the stroma was detected, the area directly under Bowman's Layer was photographed since that is where one would expect to see damage if it had occurred. Images were prepared using a Spot Insight Digital Camera and Spot 4.0.8 software (Diagnostic Instruments, Inc., Sterling Heights, MI). The color balance of the images was corrected to better represent the colors that would be seen through the microscope.

With the digital camera and associated software, it is possible to measure distances within the captured image. This feature can be used to measure corneal thickness directly. Such measurements require a true cross-section of the cornea (i.e., a perpendicular section relative to the corneal surface plane). In some cases, a true cross-section is not available. The Descemet's Membrane thickness is used to estimate how close the section is to a true cross section. The appearance of an unusually thick membrane suggests that the section was cut at a tangent to the true cross section (or a very old animal). If the measured corneal section did not appear to be a true cross section, the observation was noted. Even in a true cross section, the isolated cornea is not of uniform thickness but rather shows peaks and valleys. Therefore, an effort has been made to select "representative" cross-sections that are neither extreme "peaks" nor "valleys" for measurement of corneal thickness. An example of such a measurement is seen in Figure 5. The values obtained should be considered "representative" of the treatment group rather than strict quantitative measures. Many more measurements would be required to provide a quantitative comparison. In some cases, the treated corneas will have lost most of their epithelium or it has been compromised (i.e., ethanol treatment). In those cases, the stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

Histological Evaluation

The negative control corneas were treated for 10 minutes with sterile, deionized water (slides B7716-B7718). The negative control-treated epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. The basal cells were always tightly attached to each other. Several layers of wing cells covered the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figures 2 and 3).

The stromal elements showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining to narrow, elongated and condensed with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. In the zone directly under Bowman's Layer, there was a moderate number of keratocytes with more active nuclei (larger) and slight eosinophilic cytoplasmic staining. Collagen bundles were generally parallel and well ordered (Stroma just under Bowman's Layer, Figure 4).

The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well maintained.

A cross section of the negative control showing the general thickness of the whole cornea and stroma is provided in Figure 5.

The positive control corneas (slides B7719-B7721), treated for 10 minutes with 100% ethanol, showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei (Figure 6). The loss of adhesion between the basal cells (cell to cell) and the basal lamina was marked. The epithelium was probably not viable at the time of fixation. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 7). In the stroma directly below Bowman's Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate/marked collagen matrix vacuolization extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 8) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization (Figure 9). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 10). The endothelial cells were generally intact (similar to the negative control-treated corneas).

Table 3. Histological Evaluation

IIVS Number	Sponsor's Designation	Observations	Figure #
05AD40, Aerosol Slides B7722-B7724	CG, neat-aerosol exposure, 3-minute exposure, 120-minute post-exposure, 07/05/05	Epithelium: The surface squamous epithelium was lost or loosened in most fields. The deeper squamous, wing and basal cells were similar to the corresponding cells in the negative control-treated corneas (Figures 11 and 12). Stroma: The test article-treated corneas were similar in thickness to the negative control-treated corneas (Figure 13). No consistent increases in collagen matrix vacuolization or keratocyte changes were observed (Figure 14, directly below Bowman's Layer). Endothelium: Similar to the endothelium of the negative control-treated corneas.	11 - 14
05AD40 Aerosol	CG, neat-aerosol	Epithelium: The surface squamous epithelium was coagulated and the cytoplasmic and nuclear	15 - 19

IIVS Number	Sponsor's Designation	Observations	Figure #
Slides B7725-B7727	exposure, 10-minute exposure, 120-minute post-exposure, 07/05/05	structures lost. The deeper squamous cells (and, in a few fields, upper layer of wing cells) were largely disrupted and the nuclei and cytoplasmic staining again showed a marked blanching suggesting loss of stainable components. The deeper wing cell and basal cell layers were similar to those of the negative control-treated corneas except for a moderate increase in the fraction of cells showing increased cytoplasmic vacuolization (Figures 15 and 16). Stroma: The test article-treated corneas were slightly thicker than the negative control-treated corneas (Figure 17). There was a slight increase in collagen matrix vacuolization in the upper quarter of the stroma. The deeper stroma was generally similar the stroma of the negative control-treated corneas. There were patches of deep stroma (directly above Descemet's Membrane) that did show some increased vacuolization suggestive of loss of endothelial cell function. In the zone directly below Bowman's Layer, there was a slight increase in the frequency of keratocytes with slightly abnormal chromatin condensation and cytoplasmic eosinophilia (Figure 18). Below this zone, the keratocytes were normal (Figure 19). Endothelium: Some of the fields showed a loss of functional endothelium. However, the majority of fields showed functional endothelium. In addition, there was no damage to the deep stroma. Therefore, the loss of endothelium may have been the result of mechanical damage rather than test article exposure.	
05AD40 Instillation Slides B7728-B7730	CG, neat-installation exposure, 3-minute exposure, 120-minute post-exposure, 07/05/05	Epithelium: The surface squamous epithelium was coagulated and the cytoplasmic and nuclear structures lost. The deeper squamous cells (and, in a few fields, upper layer of wing cells) were largely disrupted and the nuclei and cytoplasmic staining again showed a marked blanching suggesting loss of stainable components. The deeper wing cells and the basal cell layer were intact except for increased cytoplasmic vacuolization in the basal cells (Figures 20 and 21). Stroma: The test article-treated corneas were	20 - 23

IIVS Number	Sponsor's Designation	Observations	Figure #
		similar in thickness to the negative control-treated corneas (Figure 22). No appreciable increase in collagen matrix vacuolization was observed. There was a slight increase in keratocytes with cytoplasmic eosinophilia in the zone directly below Bowman's Layer (Figure 23). The deeper keratocytes were essentially normal. Endothelium: Similar to the endothelium of the negative control-treated corneas.	
05AD40 Instillation Slides B7731-B7733	CG, neat-installation exposure, 10-minute exposure, 120-minute post-exposure, 07/05/05	Epithelium: The squamous epithelium was coagulated and the cytoplasmic and nuclear structures lost. The wing cell layer was largely disrupted and the nuclei and cytoplasmic staining again showed a marked blanching suggesting loss of stainable components. The basal cells were largely intact (Figures 24 and 25). Stroma: The test article-treated corneas were slightly thicker than the negative control-treated corneas (Figure 26). Slight collagen matrix vacuolization extended through the upper third of the stroma. The deeper stroma was similar to that of the negative control-treated corneas. There was some damage to the endothelium but the absence of deep stromal vacuolization suggests that the damage was mechanical and occurred after fixation. In the zone directly under Bowman's Layer, there was a slight increase in the frequency of keratocytes with slightly increased cytoplasmic vacuolization (Figure 27). Endothelium: The endothelium was intact in most fields and where it was damaged, the damage probably occurred after fixation.	24 - 27
05AD42 Instillation Slides B7746-B7748	BJ, neat-installation exposure, 3-minute exposure, 120-minute post-exposure, 07/05/05	Epithelium: Much of the squamous cell layer was lost (through coagulation and lost of the cellular contents). In a minority of fields, the full squamous layer was lost. The wing and basal layers were intact (similar to the negative control corneas) except for a slight increase in cytoplasmic vacuolization (Figures 28 and 29). Stroma: The test article-treated corneas were thicker than the negative control-treated corneas (Figure 30). Moderate collagen matrix vacuolization extended to mid depth. Below mid	28 - 31

IIVS Number	Sponsor's Designation	Observations	Figure #
		depth, the collagen matrix was normal. In the zone directly below Bowman's Layer, there was a slight to moderate increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia (Figure 31). Endothelium: Similar to the endothelium of the negative control-treated corneas.	
05AD42 Instillation Slides B7749-B7751	BJ, neat-installation exposure, 10-minute exposure, 120-minute post-exposure, 07/05/05	Epithelium: The full squamous cell and wing cell layers were coagulated and had lost must of their nuclear and cytoplasmic contents. In many fields, the basal cells showed marked nuclear and cytoplasmic vacuolization and decreased staining (blanching) suggestive of the loss of stainable components within the cells. The remaining basal cells showed marked abnormal chromatin condensation and cytoplasmic eosinophilia (Figures 32 and 33). Most of the epithelium was probably not viable at the time of fixation. Bowman's Layer was intact in all sections. Stroma: The test article-treated corneas were appreciably thicker than the negative control-treated corneas (Figure 34). Marked collagen matrix vacuolization extended to mid depth and moderate vacuolization extended well past mid depth. Where the endothelium was damaged, increased vacuolization was observed directly above Descemet's Layer. There was a marked increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia in the upper 2/3s of the stroma (Figure 35). This kind of keratocyte change is observed in corneas where the epithelium has been removed mechanically (Harbell and Curren, 2005). Thus, the change may be the result of the stromal swelling rather than direct test article action on the cells. Endothelium: The endothelium was compromised in at least half of the fields examined. The stroma above these fields showed increased vacuolization indicating that the loss of functional endothelium occurred before fixation.	32 - 35

Histological evaluation summary: Corneas exposed to CG by the aerosol route for three minutes showed only minimal changes and these changes were confined to the surface epithelium. Similar exposure for 10 minutes produced somewhat deeper damage into the upper epithelium. There was also some slight stromal swelling directly under the epithelium. Keratocyte changes were minimal. Corneas exposed to CG by the instillation route for three minutes again showed only changes confined to the epithelium. The increased opacity score was the result of the surface coagulation. The stroma was undamaged. Exposure for 10 minutes produced deeper epithelial damage but only minimal changes in the upper stroma. The deeper stroma was normal. These data suggest that test article CG would produce minimal damage by the aerosol route and only slight damage by the instillation route. The reference substance BJ was exposed to the corneas by the installation route. The corneas treated for 3 minutes showed some coagulation in the surface epithelium and some increased cytoplasmic vacuolization in the deeper epithelium. The upper stroma showed moderate vacuolization and a moderate increase in keratocytes with enlarged nuclei and cytoplasmic eosinophilia. These stromal changes may reflect the reduction of epithelial barrier integrity that allowed water to enter the stroma rather than direct action of the reference substance on the stroma. The 10-minute exposure produced appreciable epithelial damage and resulting loss of epithelial barrier integrity. Stromal changes included marked collagen matrix vacuolization and a marked increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia. Again, these keratocyte changes may reflect the impact of stromal swelling rather than direct action of the reference substance on the cells themselves[8]. These corneas also showed some loss of endothelial cells. These data suggest that the reference substance has more eye irritation potential than does the test substance when compared using the instillation route of exposure.

The figures displayed on the subsequent pages of this report are representative hematoxylin and eosin-stained cross-sections presented at the indicated magnification. The black bar, on each micrograph, represents 100 µm. Arrows from the text to the figures are intended to show examples of the lesions mentioned. Not all lesions are marked. The vertical double-headed arrows mark the limits of the epithelium in the appropriate figures.

[8] Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. ALTEX 42(Special Issue):236.

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (magnification 290x)

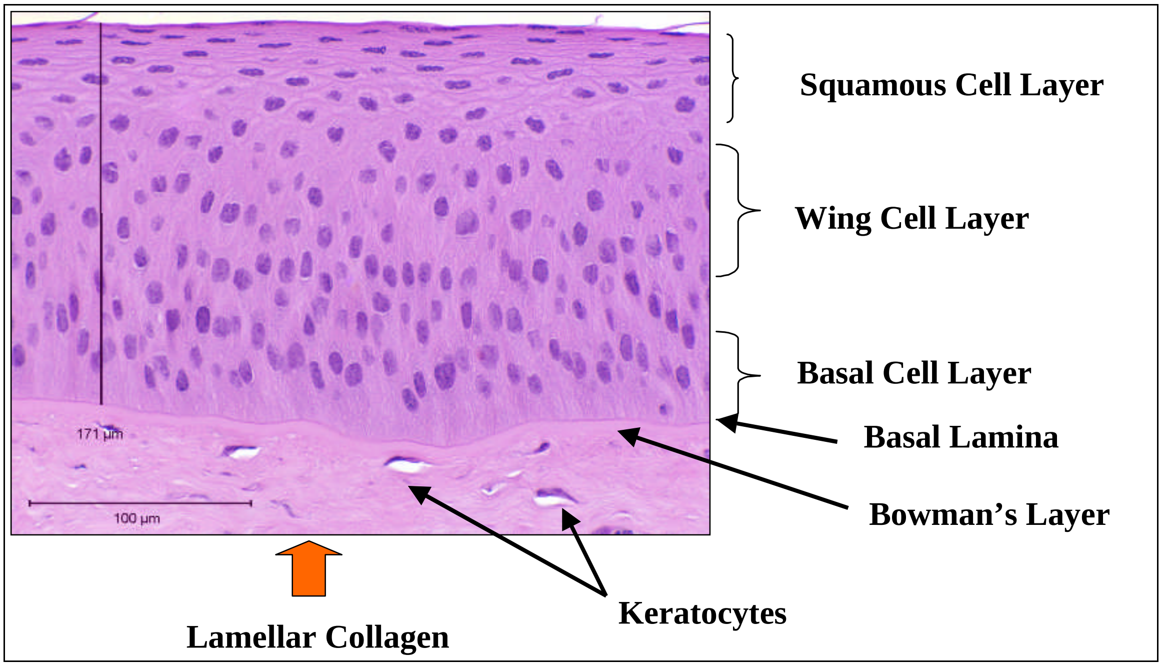


Figure 2. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

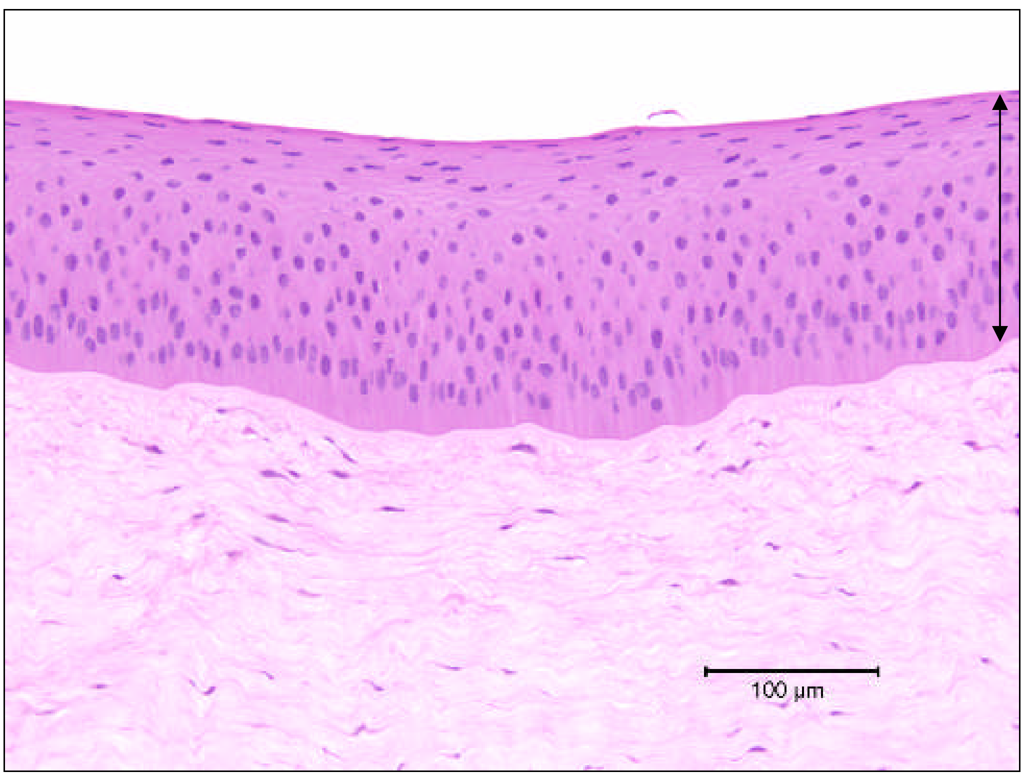


Figure 3. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (detail) (magnification 475x)

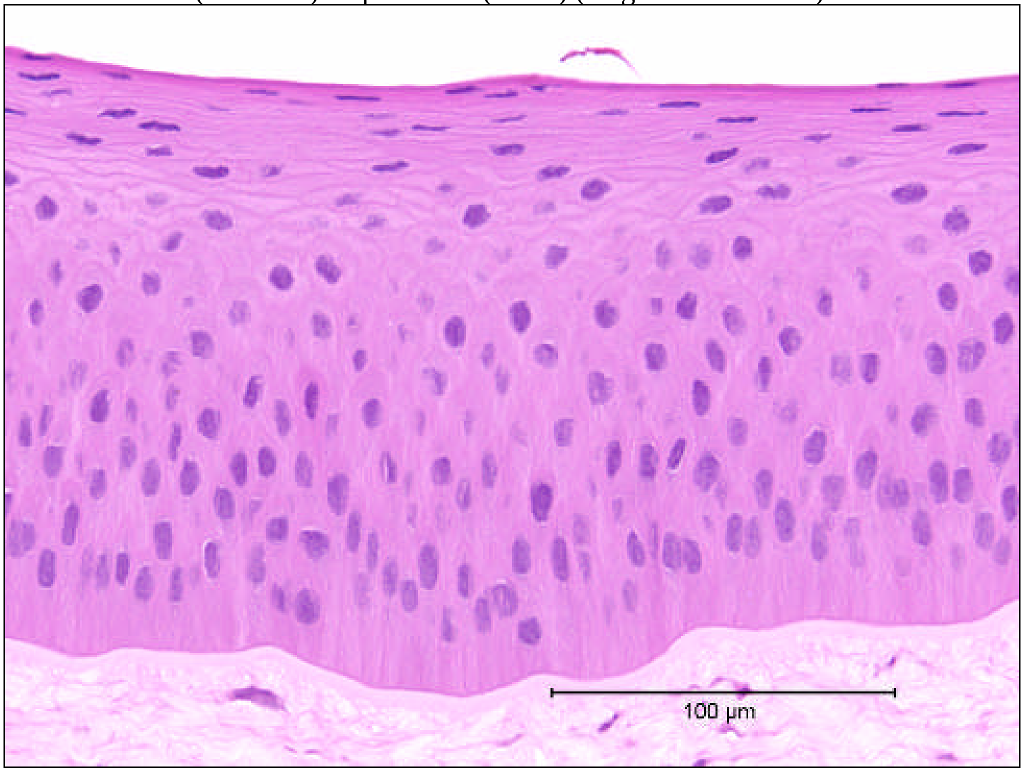


Figure 4. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer (magnification 475x)

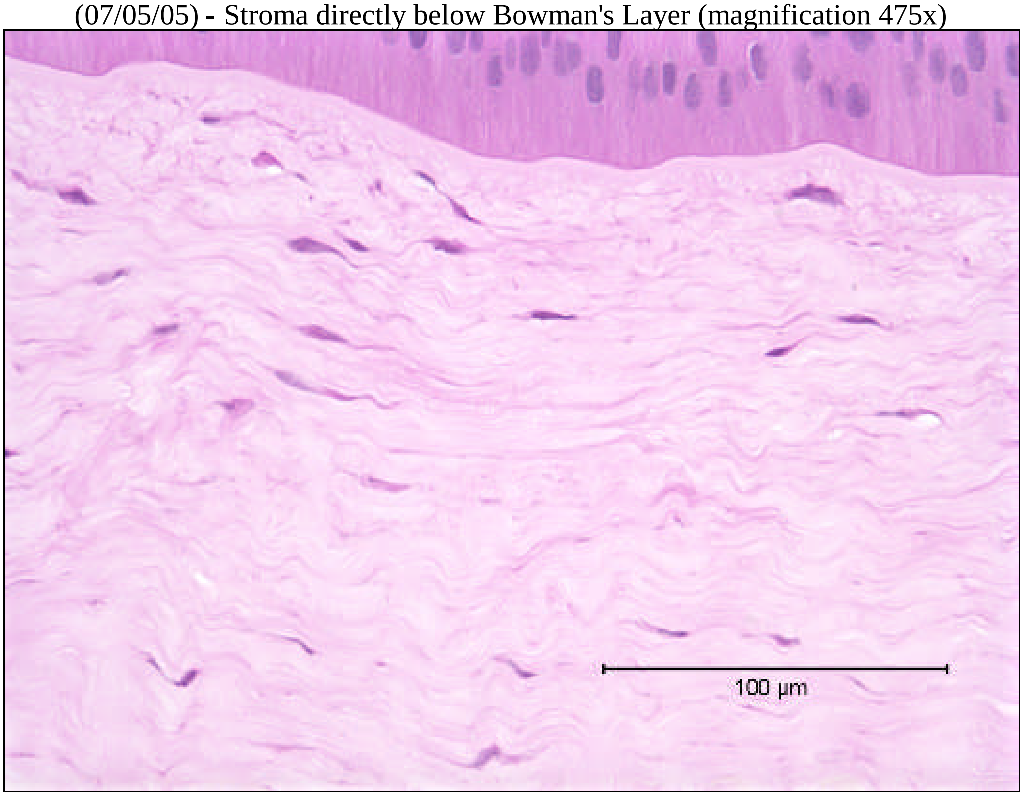


Figure 5. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

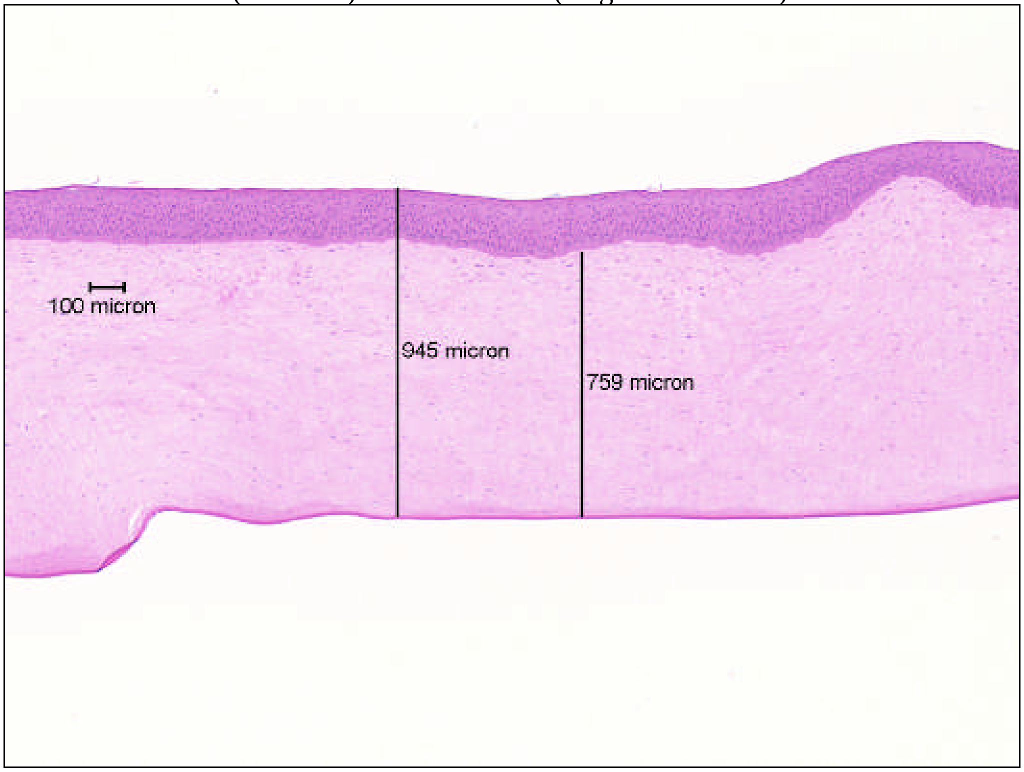


Figure 6. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (probably not viable at the time of fixation) (magnification 237x)

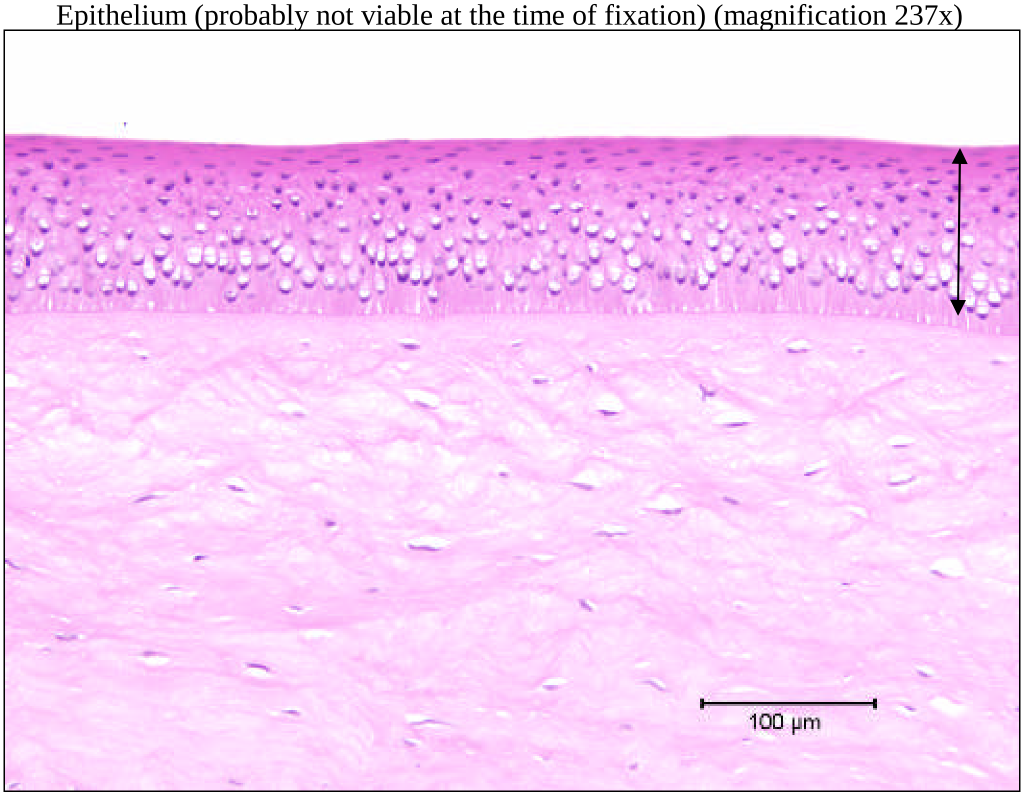


Figure 7. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

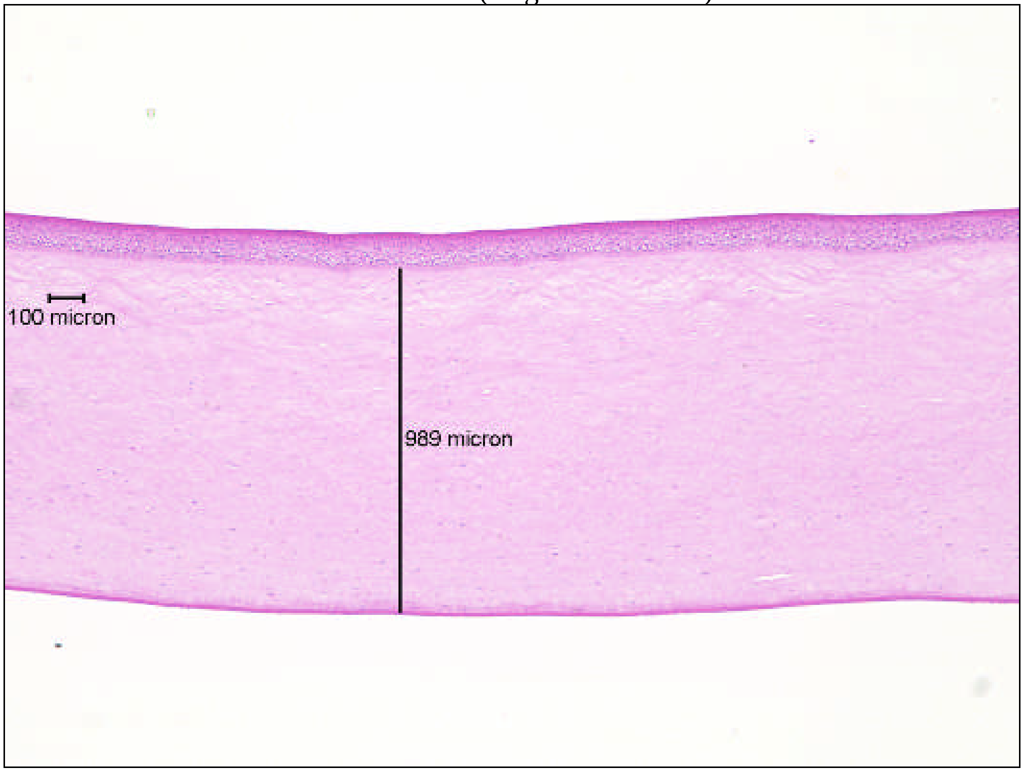


Figure 8. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Upper stroma showing hyperchromic staining in the zone directly below Bowman's Layer and the decrease in the density of viable keratocytes (magnification 237x)

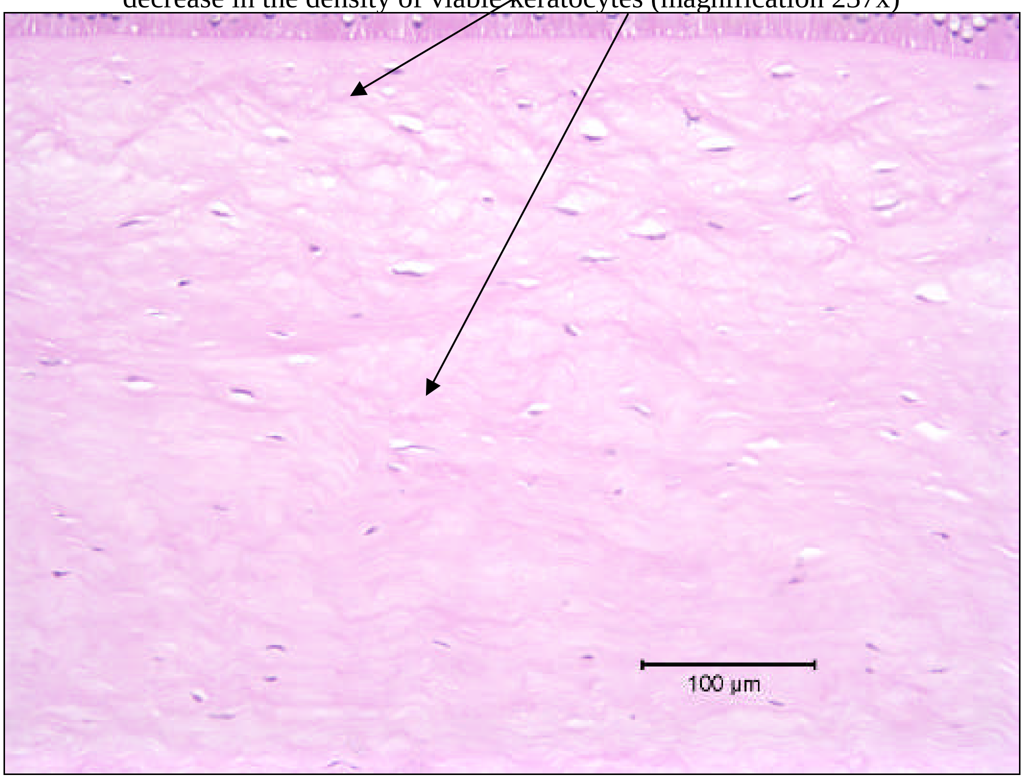


Figure 9. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and an increased frequency of keratocytes with abnormal chromatin condensation (magnification 475x)

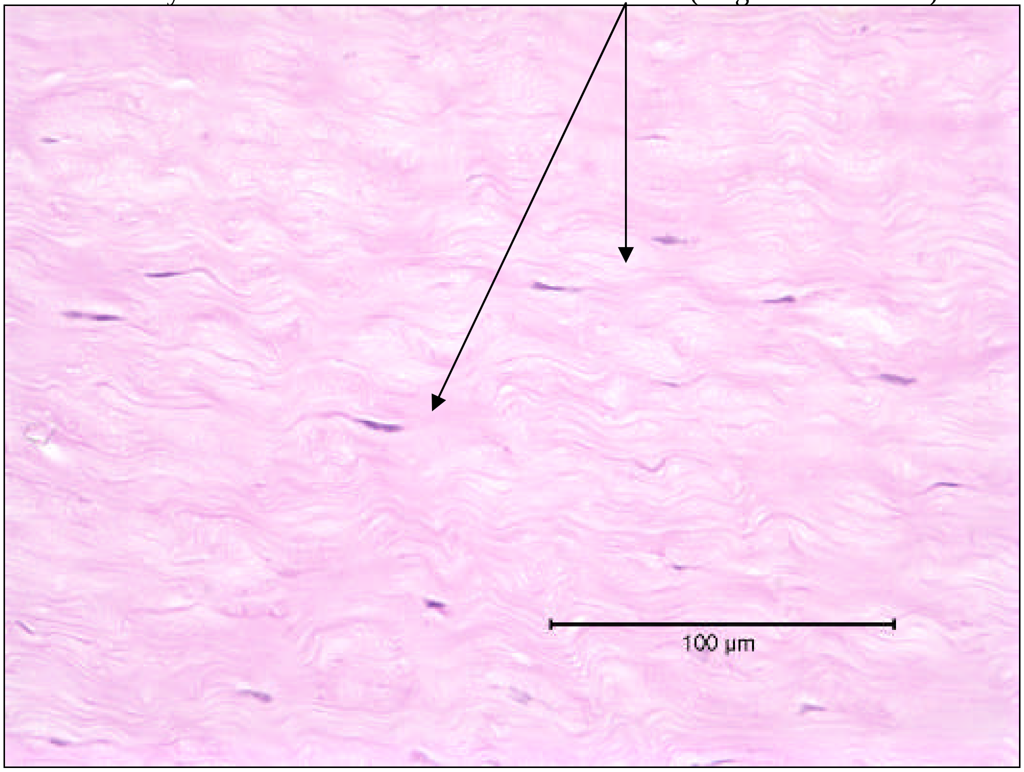


Figure 10. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma near mid depth showing keratocyte nuclear enlargement and cytoplasmic eosinophilia (magnification 475x)

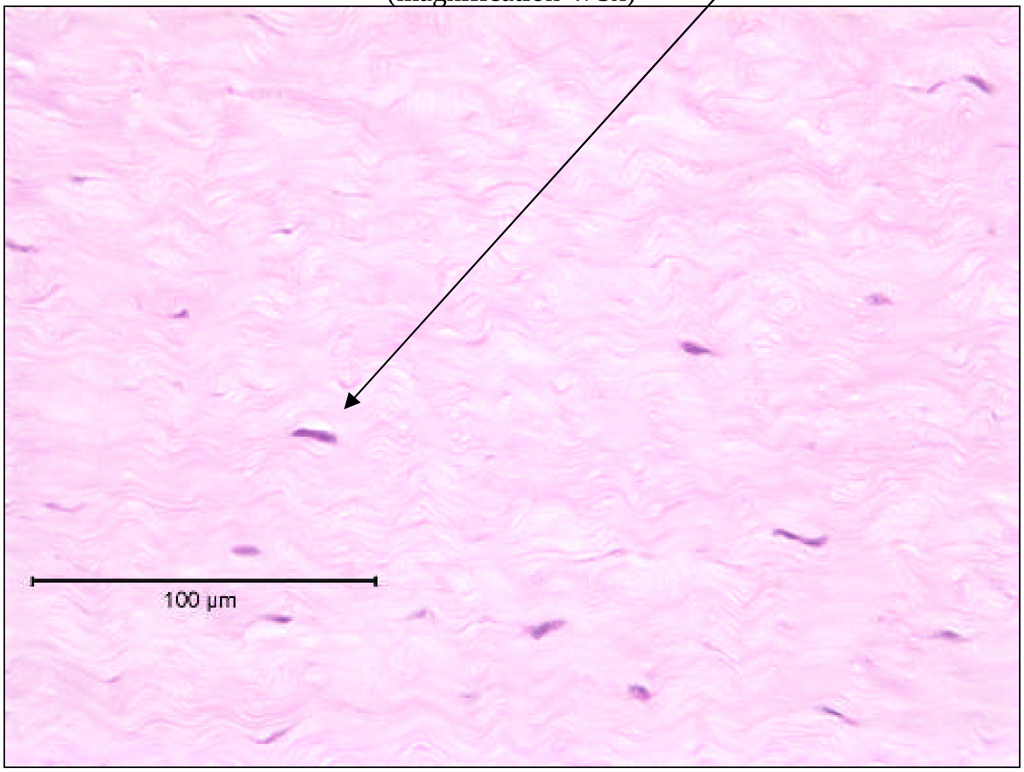


Figure 11. CG, neat- aerosol exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

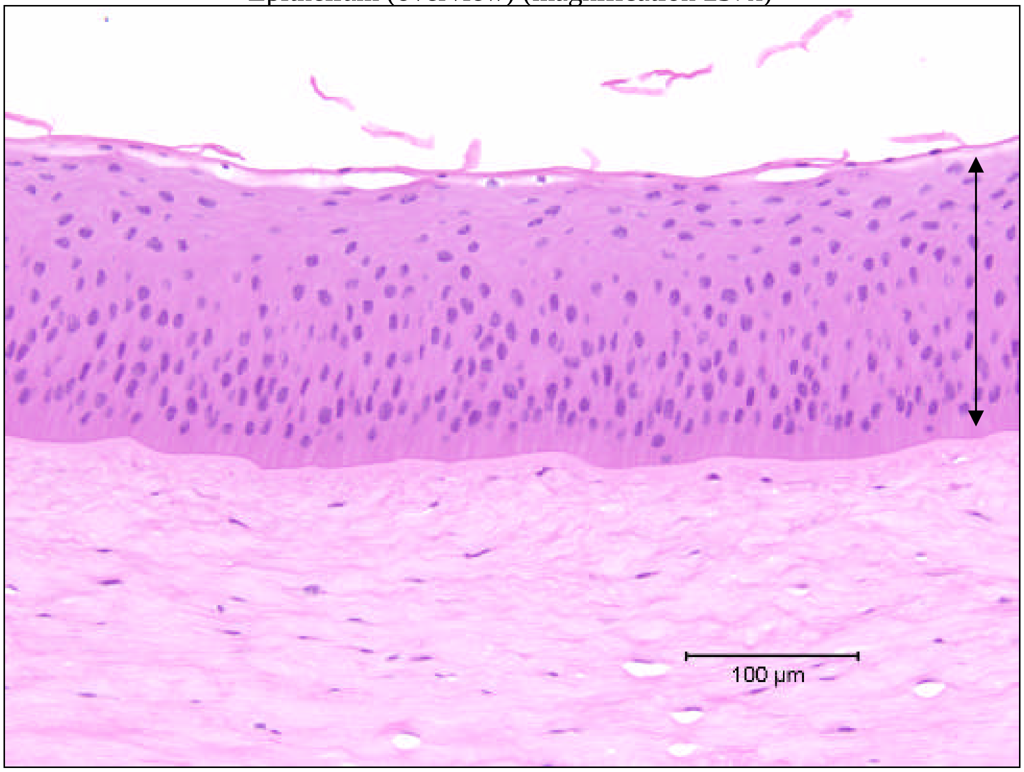


Figure 12. CG, neat- aerosol exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium showing the loss of some of the surface squamous epithelium (magnification 475x)

Figure 13. CG, neat- aerosol exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

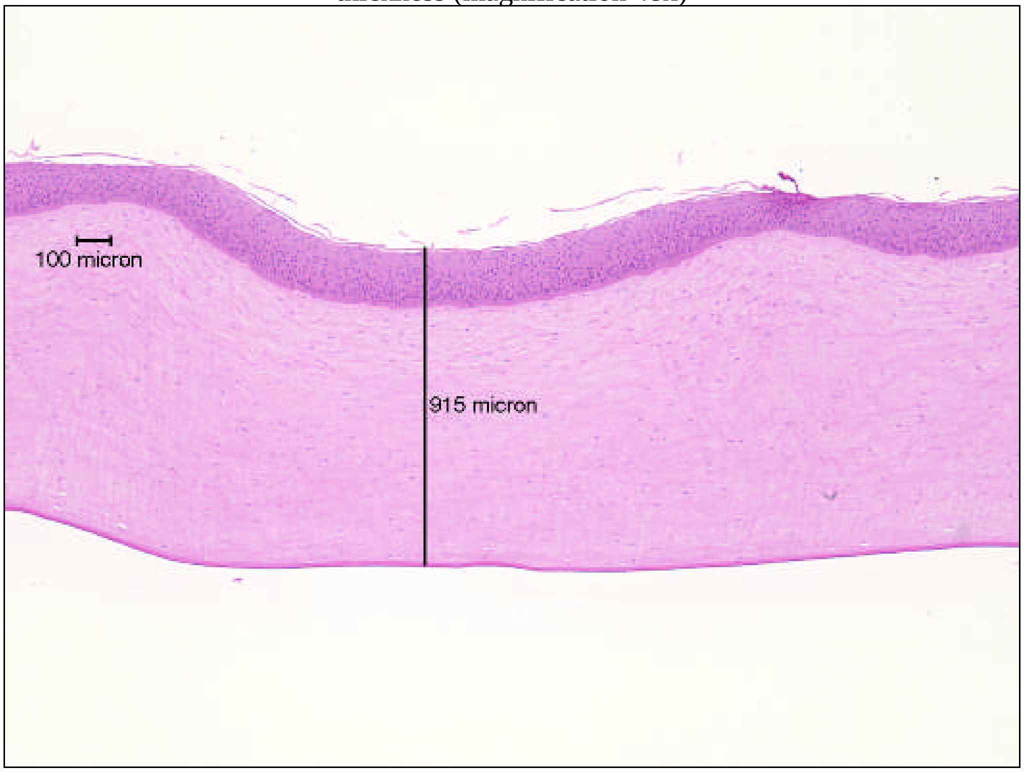


Figure 14. CG, neat- aerosol exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer (magnification 475x)

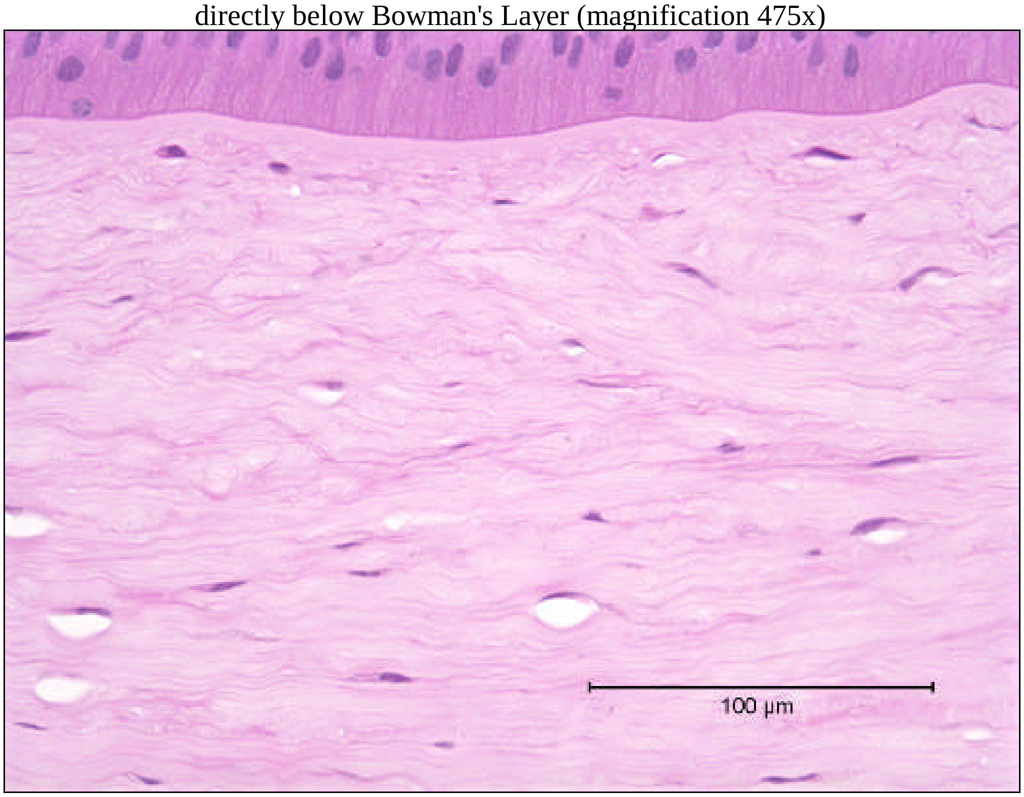


Figure 15. CG, neat- aerosol exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

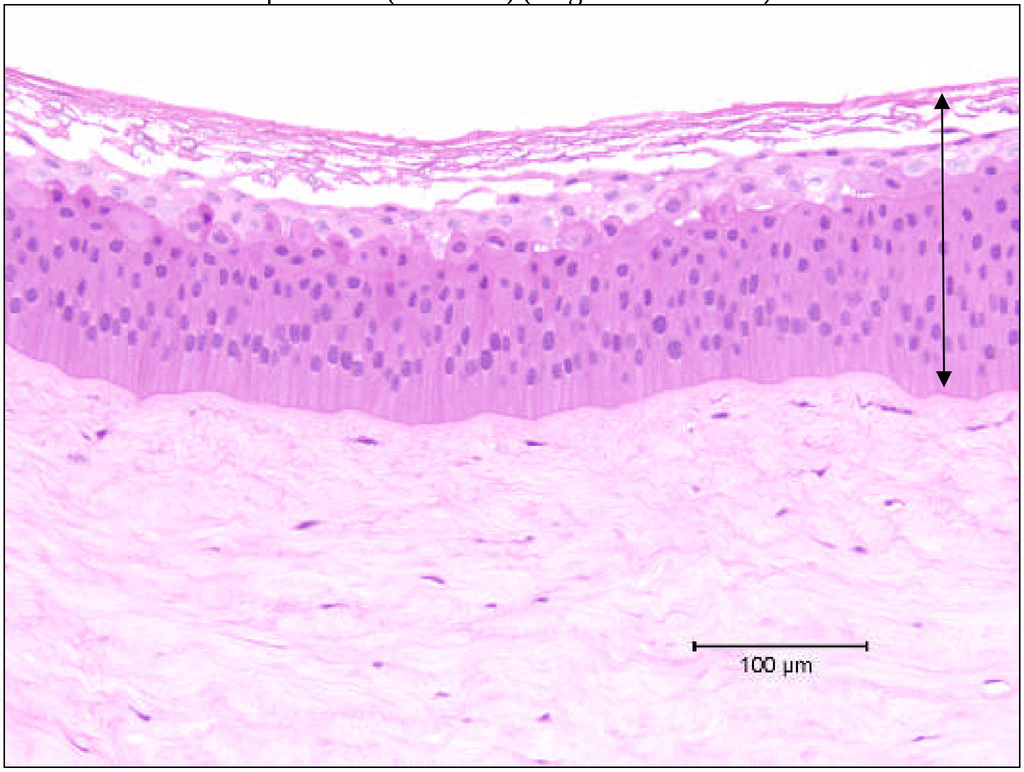


Figure 16. CG, neat- aerosol exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium showing coagulation of the upper squamous cells, disruption and blanching in the deeper squamous and increased cytoplasmic vacuolization in some of the deep wing and basal cells (magnification 475x)

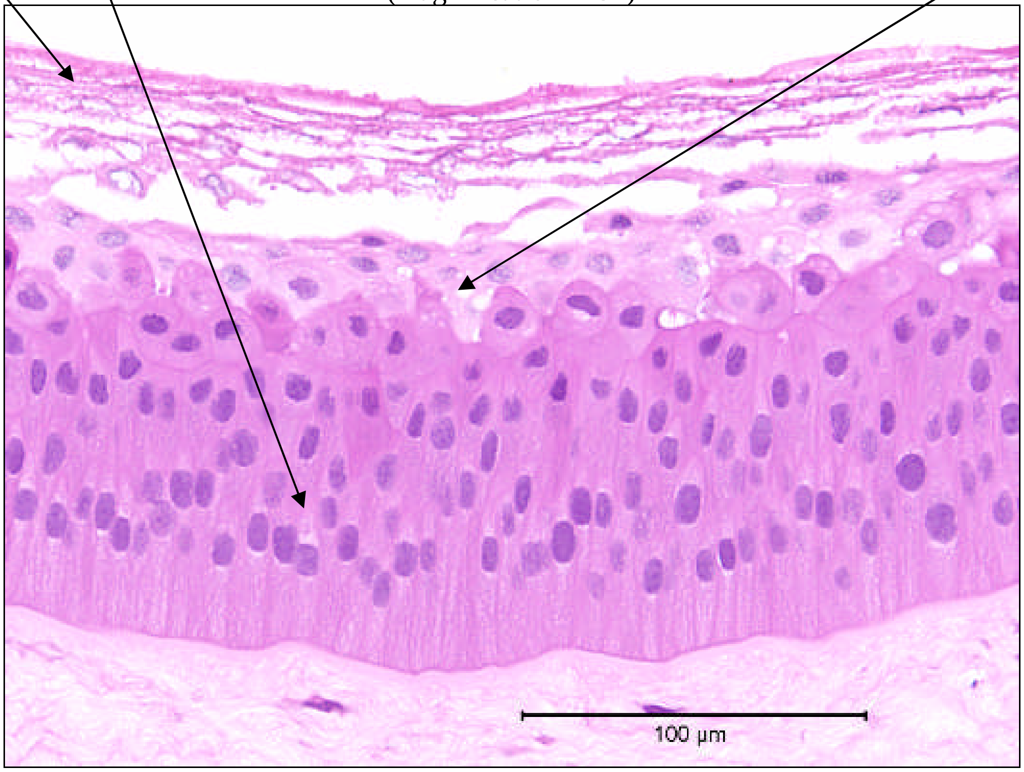


Figure 17. CG, neat- aerosol exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Full

thickness (magnification 48x)

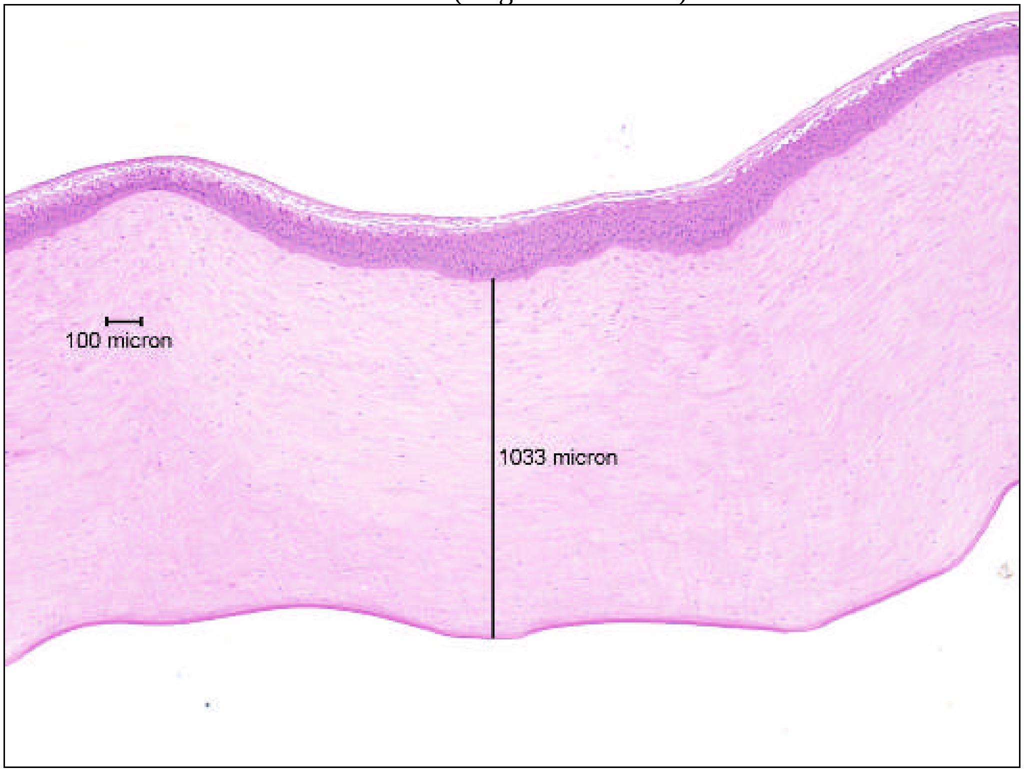


Figure 18. CG, neat- aerosol exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer showing slight collagen matrix vacuolization and a slight increase in keratocytes with abnormal chromatin condensation and cytoplasmic eosinophilia (magnification 475x)

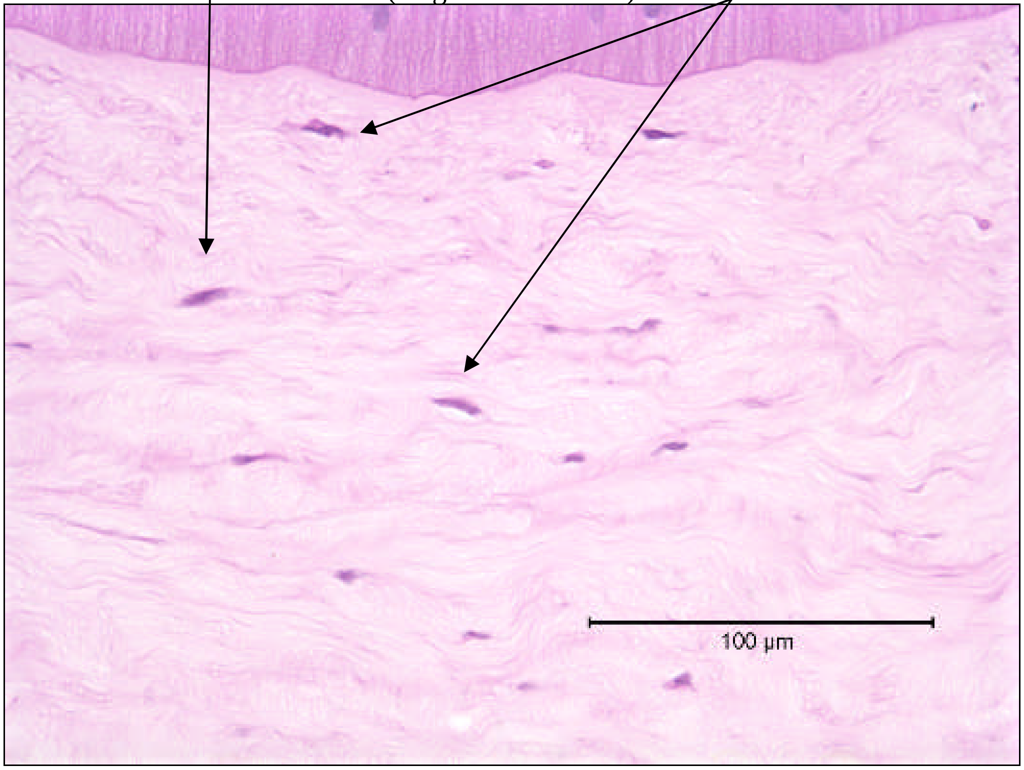


Figure 19. CG, neat- aerosol exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Essentially normal stroma below mid depth (magnification 475x)

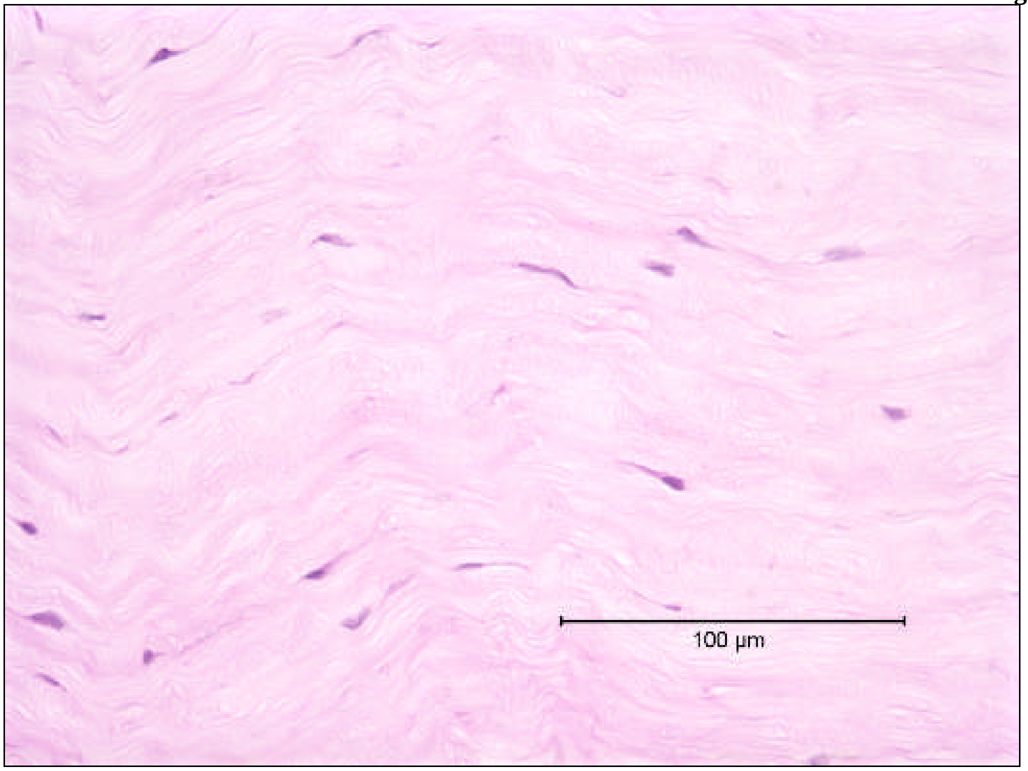


Figure 20. CG, neat- instillation exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

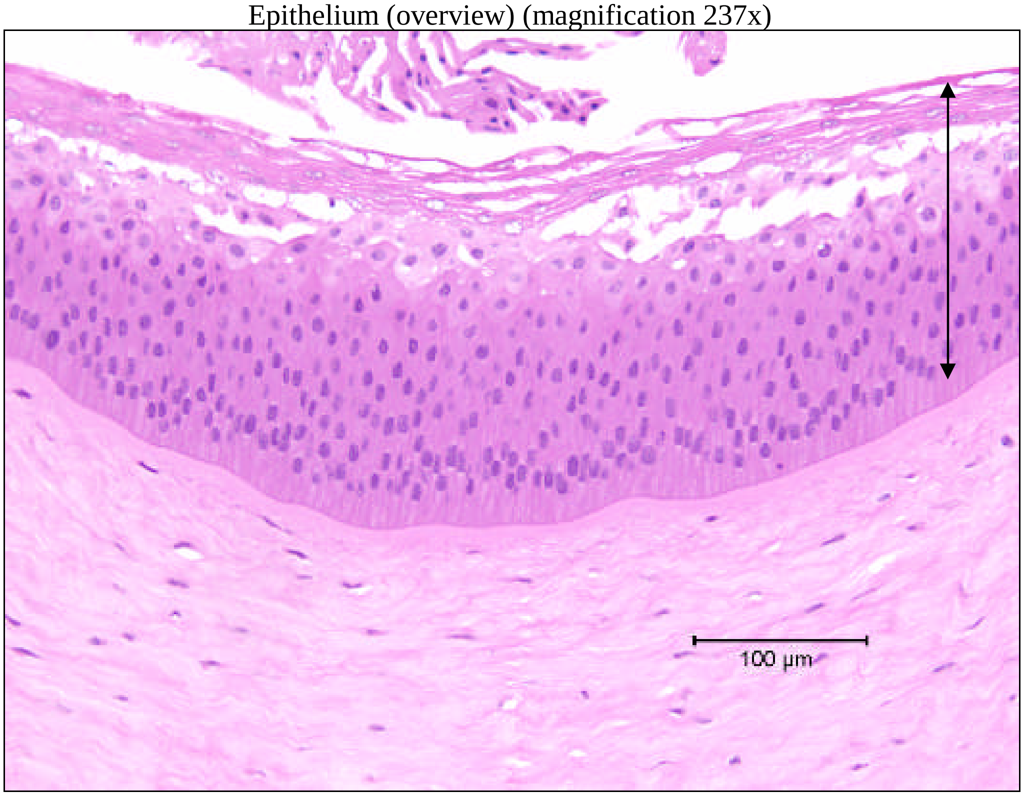


Figure 21. CG, neat- instillation exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium showing coagulation of the upper squamous cells, disruption and blanching in the deeper squamous and increased cytoplasmic vacuolization in some of the deep wing and basal cells (magnification 475x)

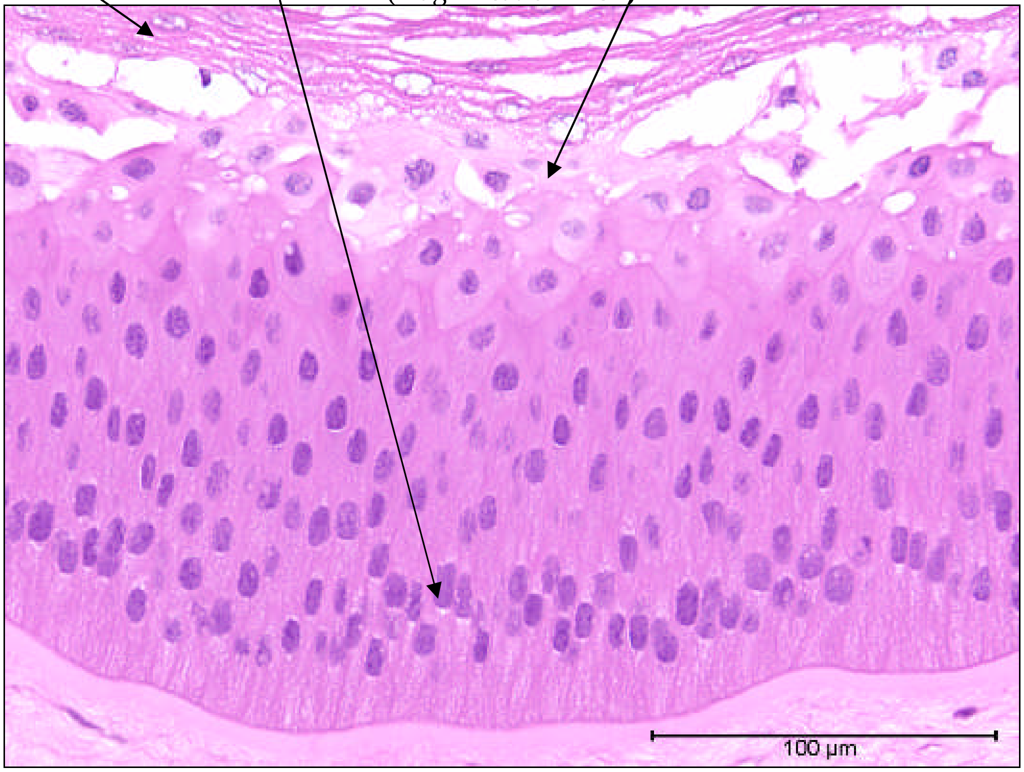


Figure 22. CG, neat- instillation exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

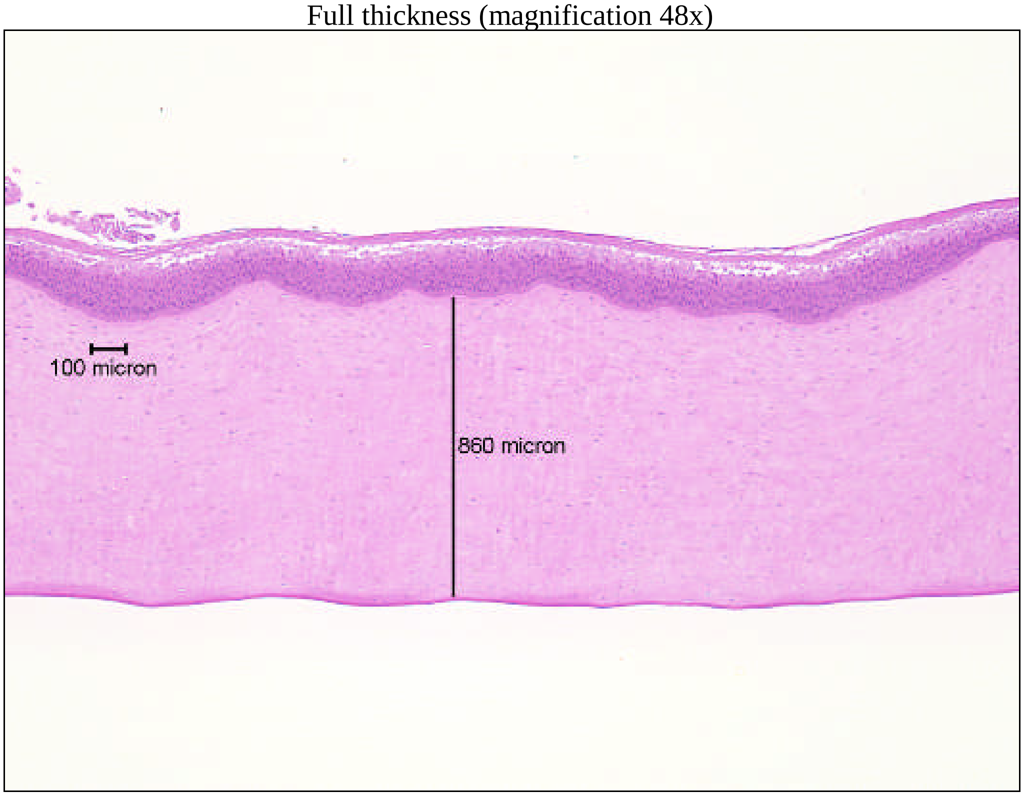


Figure 23. CG, neat- instillation exposure, 3-minute exposure, 120-minute post-exposure (07/05/05) -

Stroma directly below Bowman's Layer (magnification 475x)

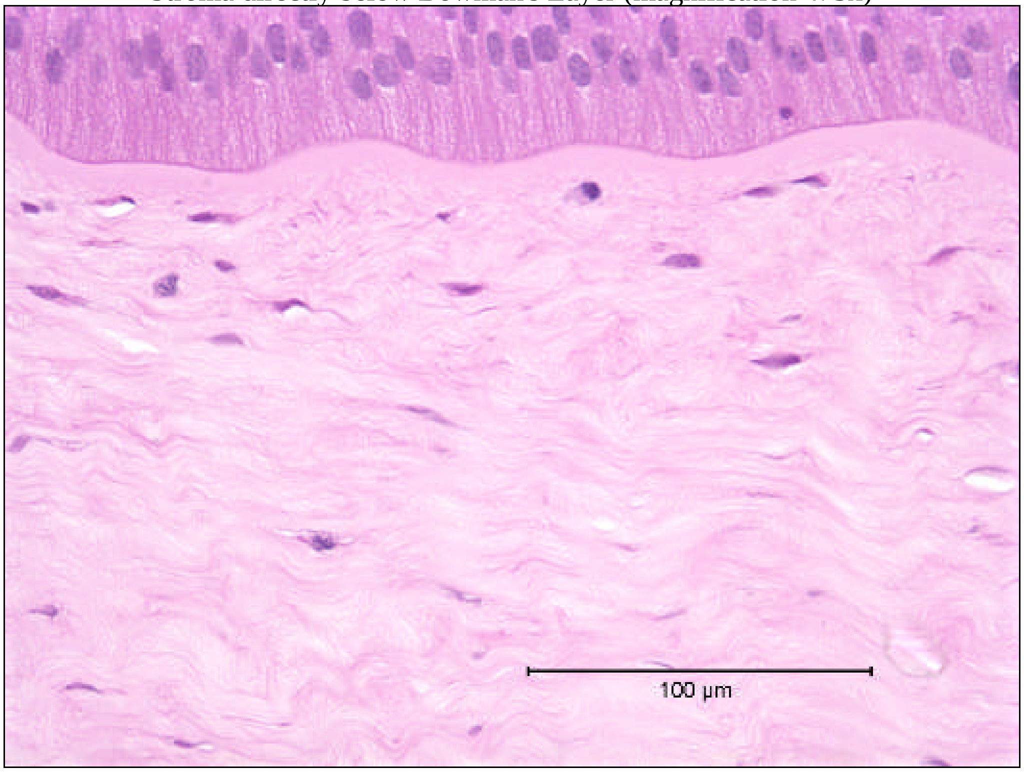


Figure 24. CG, neat-installation exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

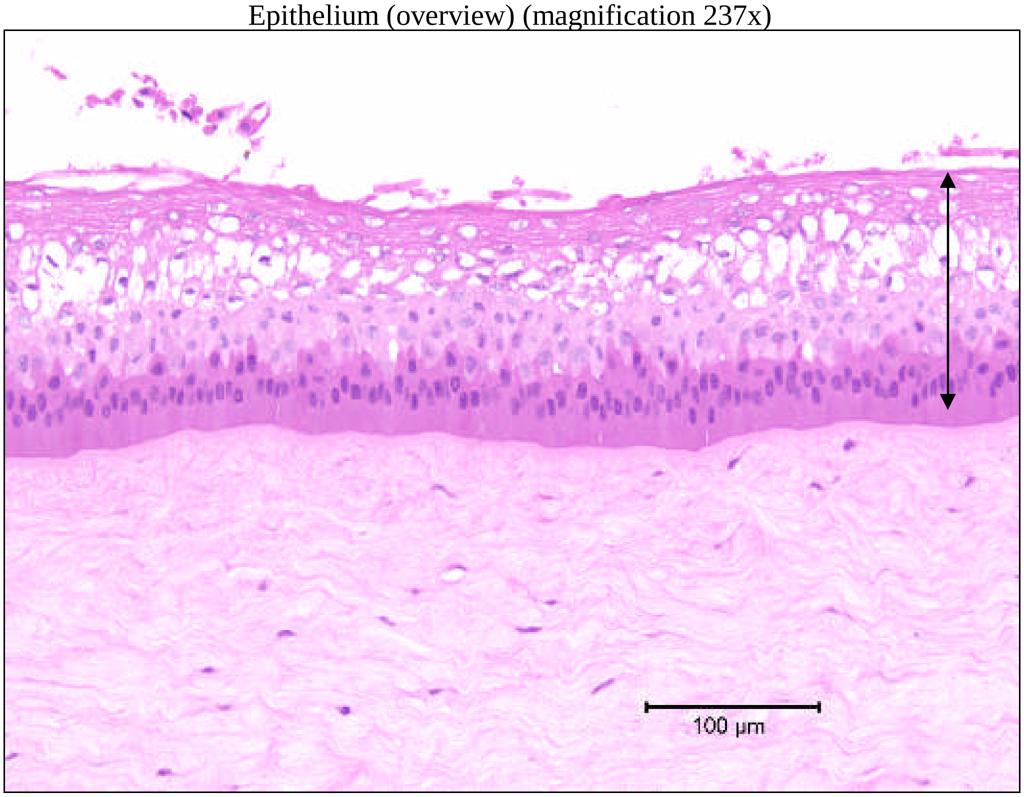


Figure 25. CG, neat-installation exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium showing coagulation of the squamous layer, blanching of the wing cell layer, and an intact basal layer (magnification 475x)

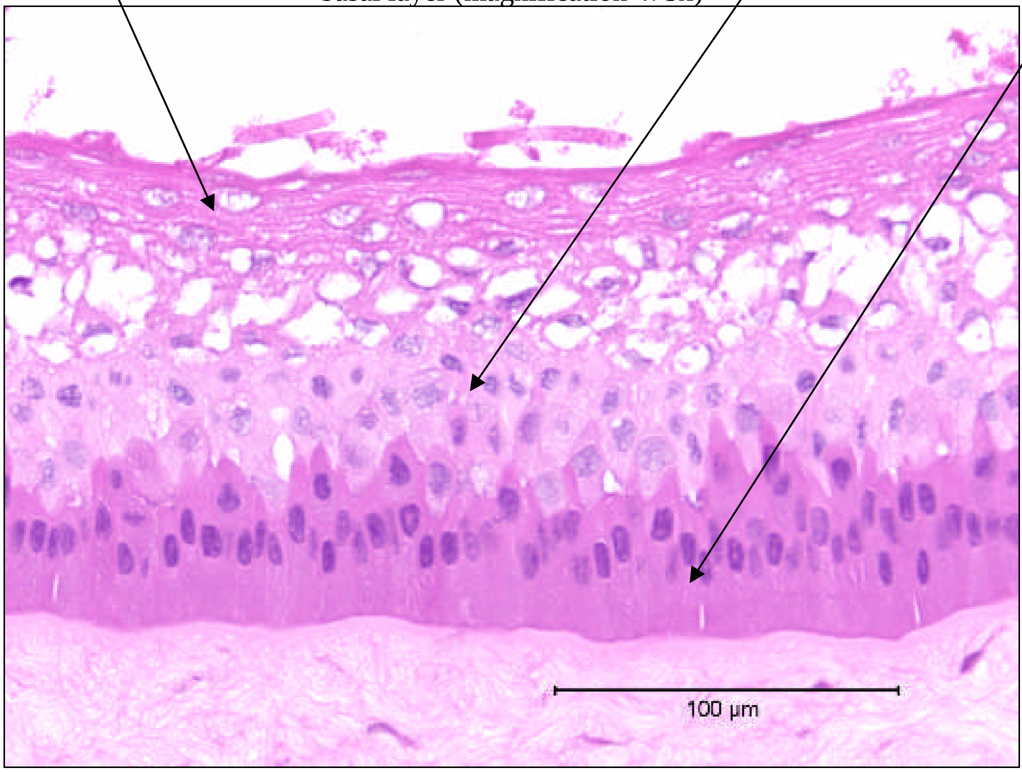


Figure 26. CG, neat-installation exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

Figure 27. CG, neat-installation exposure, 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer showing slight collagen matrix vacuolization and a slight

increase in the number of keratocytes with slight cytoplasmic eosinophilia (magnification 475x)

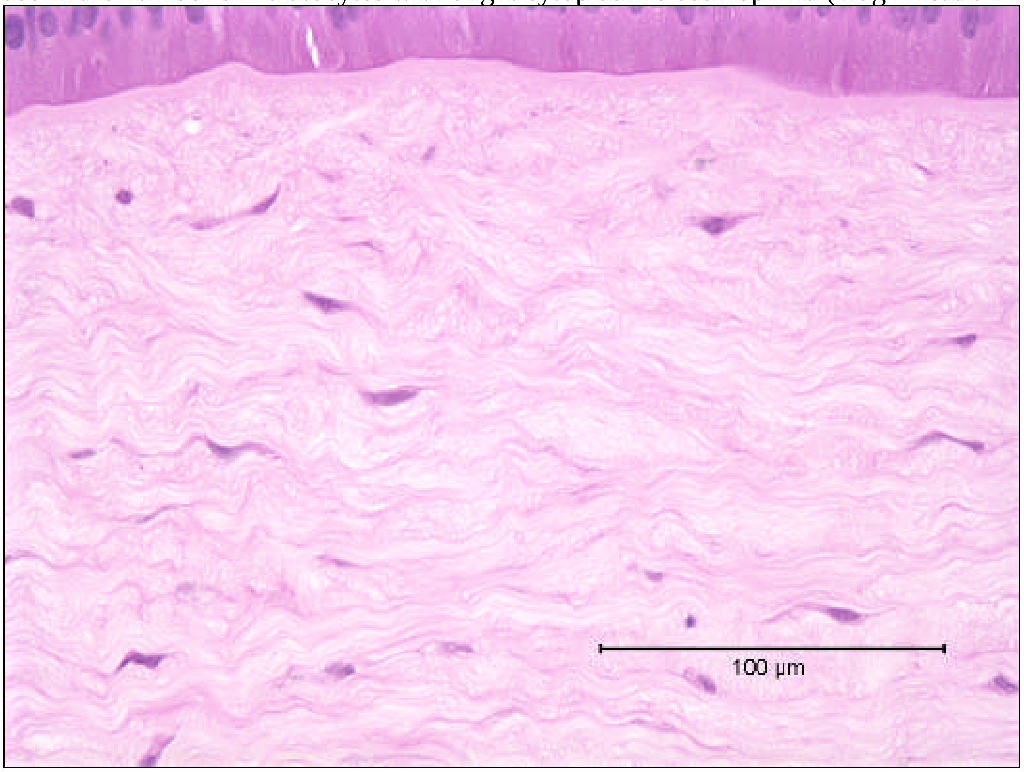


Figure 28. BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

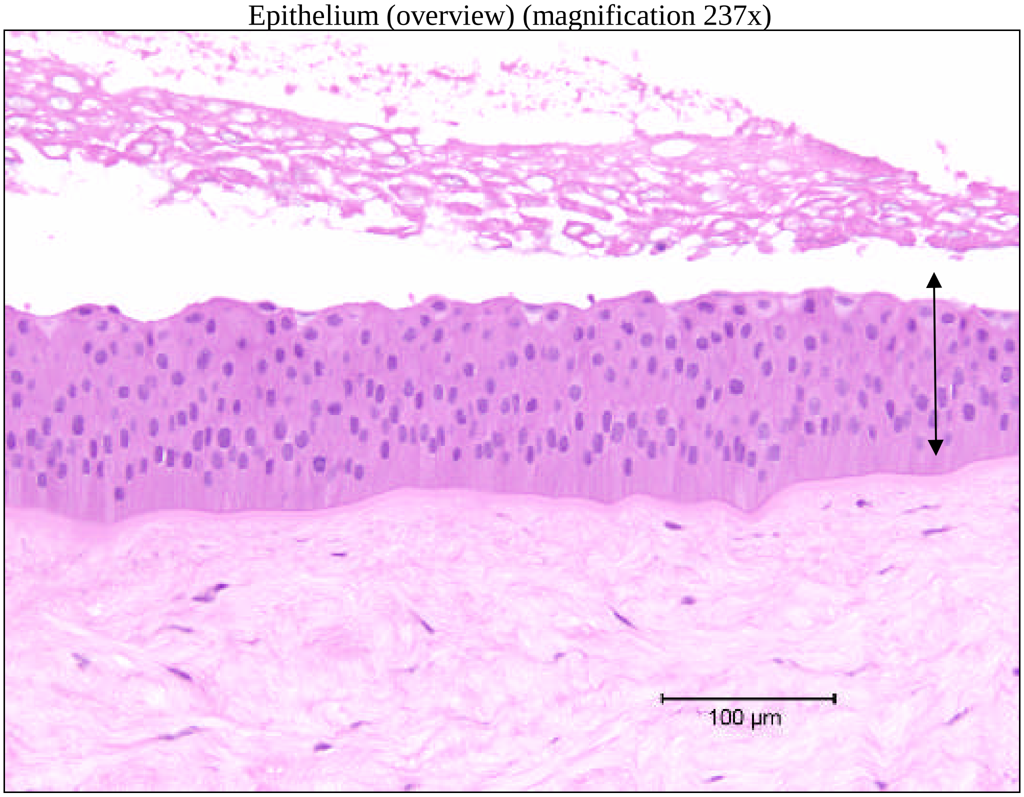


Figure 29.BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium showing the loss of the squamous cell layer and an increase in the number of wing and basal cells with cytoplasmic vacuolization (magnification 475x)

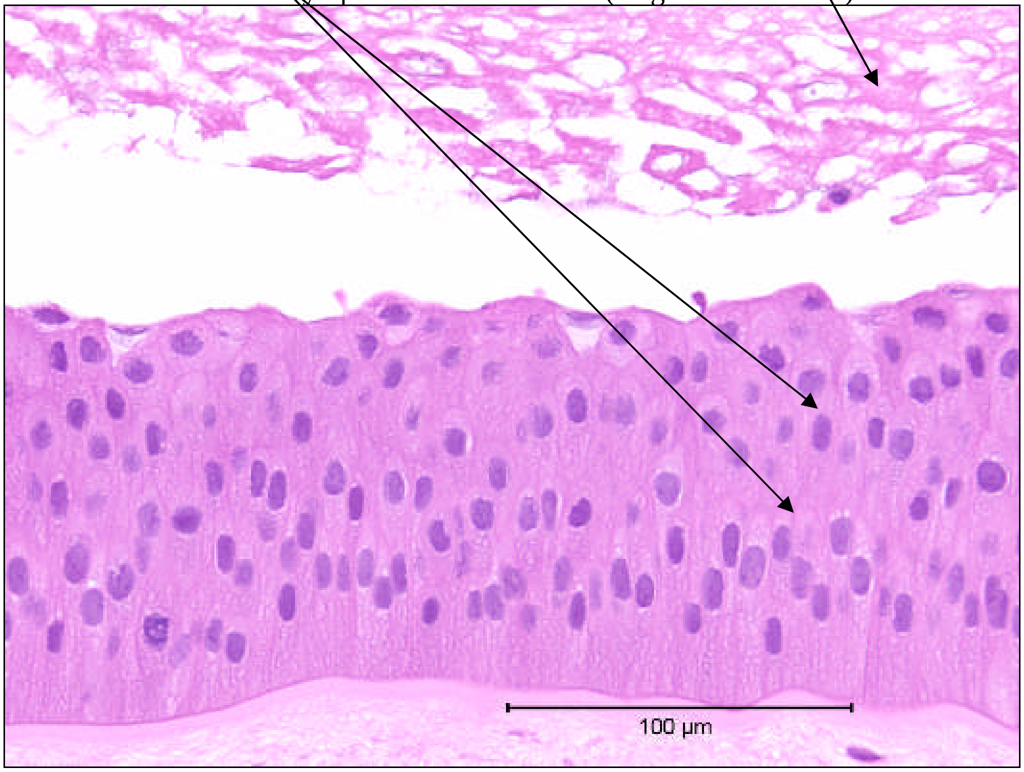


Figure 30.BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Full thickness (magnification 48x)

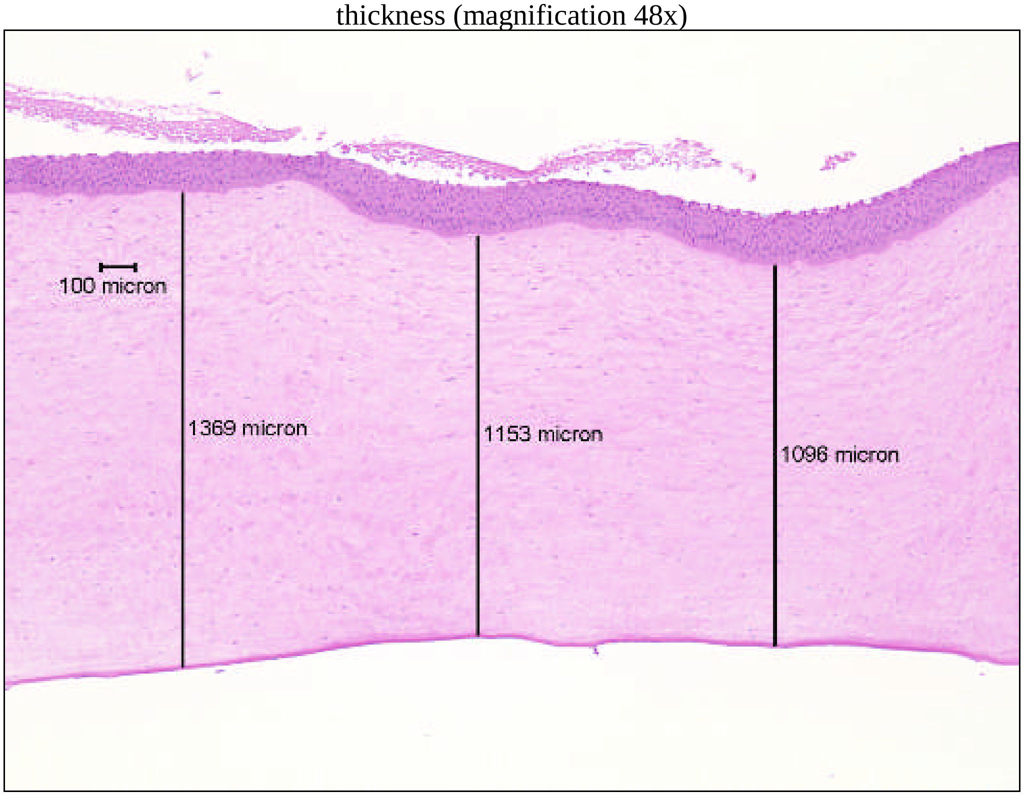


Figure 31.BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer showing moderate collagen matrix vacuolization and an increased number of keratocytes with nuclear enlargement and cytoplasmic eosinophilia (magnification 475x)

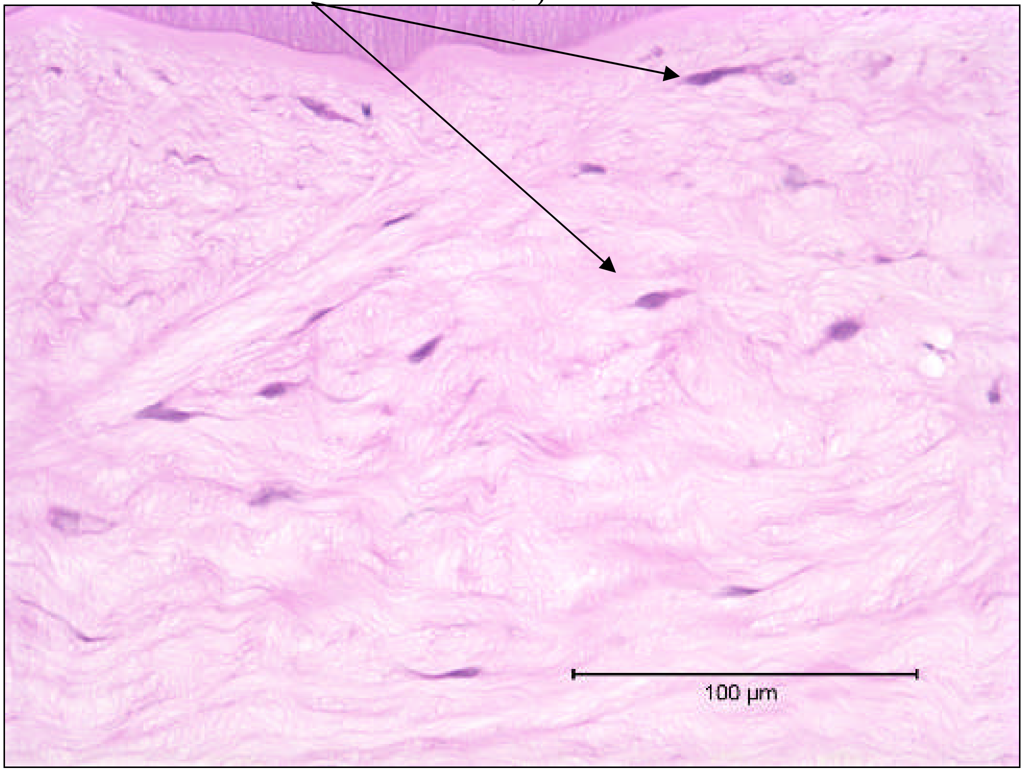


Figure 32.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

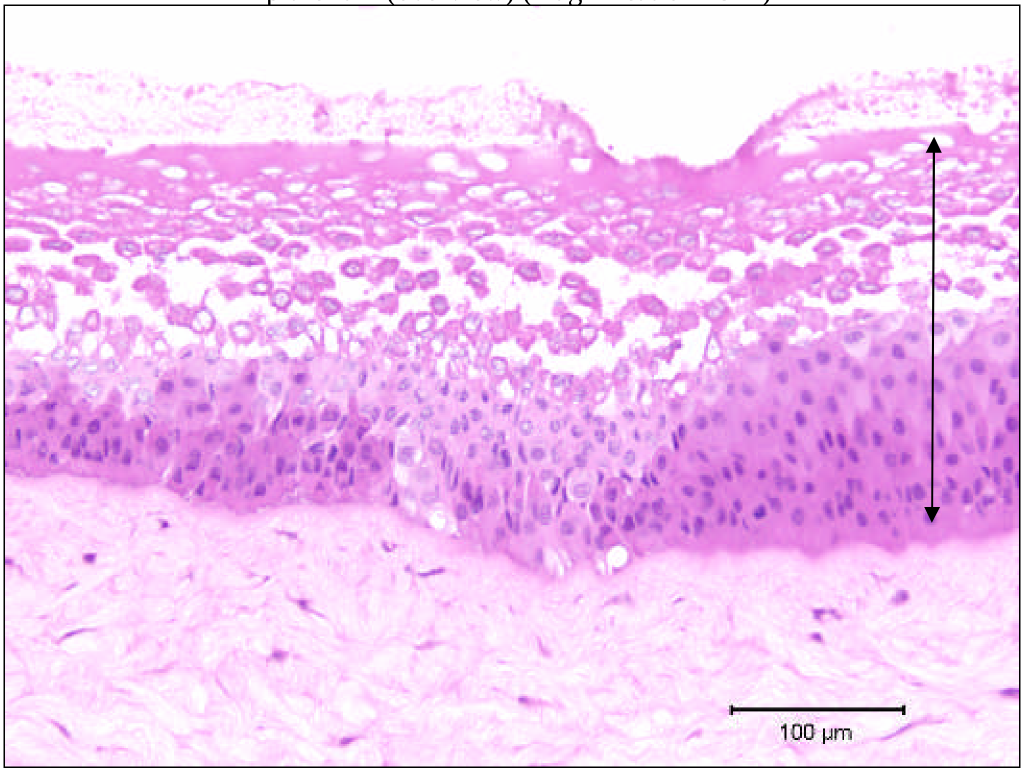


Figure 33.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium showing coagulation/loss of the squamous and wing cell layers, blanching of parts of the basal layer and abnormal chromatin condensation and cytoplasmic eosinophilia in the remaining basal cells (magnification 475x)

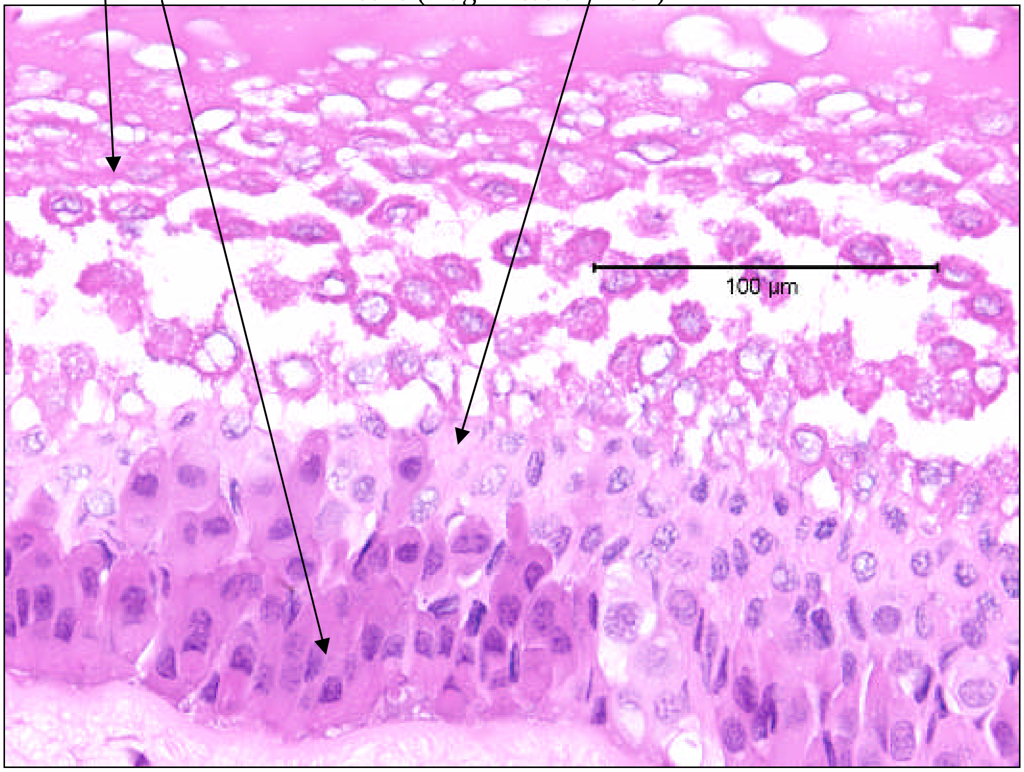


Figure 34.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Full thickness (magnification 48x)

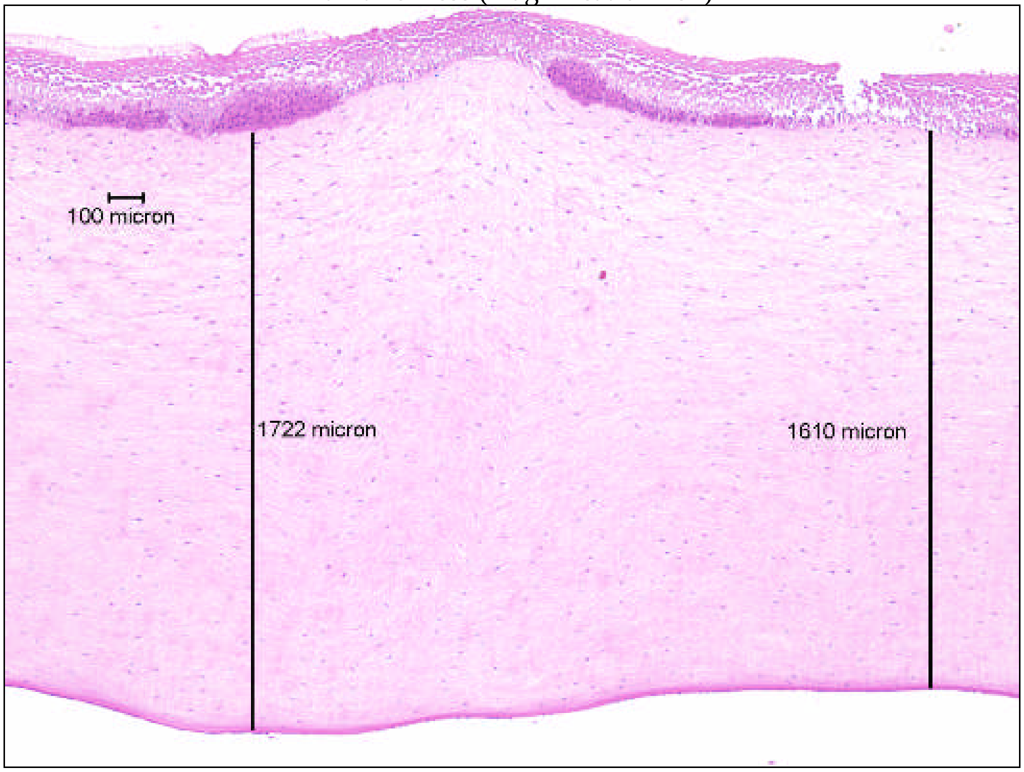
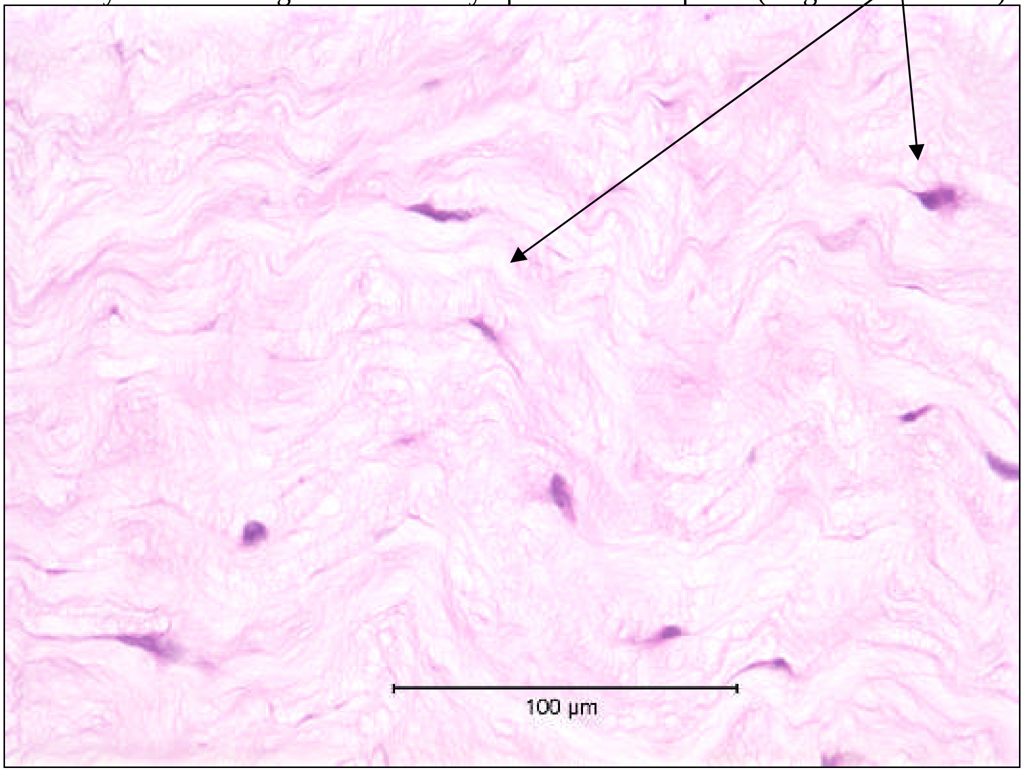


Figure 35.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Stroma at 20% depth showing marked collagen matrix vacuolization and a marked increase in keratocytes with enlarged nuclei and cytoplasmic eosinophilia (magnification 475x)



APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

~~TA #~~	~~CORNEA #~~	~~INITIAL~~	~~FINAL~~	~~CHANGE~~	~~CORRECTED~~	~~AVG~~	~~STDEV~~
05AD40-A	9	1	5	4	4.3		
Neat	10	4	6	2	2.3		
3 minutes	11	3	6	3	3.3	3.3	1.0
05AD40-A	12	5	10	5	5.3		
Neat	13	2	4	2	2.3		
10 minutes	14	4	7	3	3.3	3.7	1.5
05AD40-I	15	3	25	22	22.3		
Neat	16	3	26	23	23.3		
3 minutes	17	2	25	23	23.3	23.0	0.6
05AD40-I	18	3	29	26	26.3		
Neat	19	4	33	29	29.3		
10 minutes	20	3	34	31	31.3	29.0	2.5
05AD42-I	34	3	11	8	8.3		
Neat	35	3	7	4	4.3		
3 minutes	37	5	5	0	0.3	4.3	4.0
05AD42-I	38	4	35	31	31.3		
Neat	39	4	36	32	32.3		
10 minutes	40	4	27	23	23.3	29.0	4.9
Neg. Control	1	4	3	-1	NA		
Sterile, DI water	2	4	4	0	NA		
10 minutes	3	4	4	0	NA	-0.3	
Pos. Control	4	3	36	33	33.3		
Ethanol	6	4	34	30	30.3		
10 minutes	8	2	34	32	32.3	32.0	1.5
	*21	4					
	*22	4					
	*23	5					
	*24	3					
	*25	4					
	*26	4					
	*27	2					
	*28	4					
	*29	3					
	*30	4					
	*31	5					
	*32	5					
	*33	3					
	*36	6					
	*41	4					
	*42	4					
	*45	3					
	*48	3					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
10 minutes

Cornea #	OD490
1	0.006
2	0.006
3	0.005
Avg.	0.006

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.314	1	1.308
6	1.179	1	1.173
8	1.379	1	1.373
		Avg. =	1.285
		STDEV =	0.102

05AD40-A
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
9	0.012	1	0.006
10	0.012	1	0.006
11	0.018	1	0.012
		Avg. =	0.008
		STDEV =	0.003

05AD40-A
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
12	0.033	1	0.027
13	0.007	1	0.001
14	0.020	1	0.014
		Avg. =	0.014
		STDEV =	0.013

05AD40-I
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
15	0.107	1	0.101
16	0.063	1	0.057
17	0.291	1	0.285
		Avg. =	0.148
		STDEV =	0.121

05AD40-I
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
18	0.330	5	1.644
19	0.863	1	0.857
20	1.325	1	1.319
		Avg. =	1.274
		STDEV =	0.395

05AD42-I
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
34	0.366	1	0.360
35	0.264	1	0.258
37	0.162	1	0.156
		Avg. =	0.258
		STDEV =	0.102

05AD42-I
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
38	1.381	1	1.375
39	1.412	1	1.406
40	0.467	5	2.329
		Avg. =	1.704
		STDEV =	0.542

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AD40-A	Neat	3 minutes	3.3	0.008	3.5
05AD40-A	Neat	10 minutes	3.7	0.014	3.9
05AD40-I	Neat	3 minutes	23.0	0.148	25.2
05AD40-I	Neat	10 minutes	29.0	1.274	48.1
05AD42-I	Neat	3 minutes	4.3	0.258	8.2
05AD42-I	Neat	10 minutes	29.0	1.704	54.6
Ethanol	Neat	10 minutes	32.0	1.285	51.3